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THE BIOCHEMICAL JOURNAL

Edited for

THE BIOCHEMICAL SOCIETY

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PROCEEDINGS OF THE BIOCHEMICAL SOCIETY

The **225th Meeting** *of the Biochemical Society was held at King's College, Strand, London, W.C. 2, on Saturday, December 19th, 1942, at 2 p.m., when the following papers were read:*

The Fluorescence of the Tissues in Avitaminosis E. By T. Moore and Y. L. Wang

It has been previously reported that the uteri of rats restricted to a diet deficient in vitamin E show a marked brown discoloration.* Exposure to the irradiation of a mercury arc lamp, screened with Wood's glass, has now been found to evoke a characteristic yellow or bronze-coloured fluorescence. This fluorescence allows a much more delicate test for vitamin-E deficiency than the inspection of the affected tissues in visible light. Thus in early stages of deficiency strong yellow fluorescence is exhibited before the appearance of noticeable discoloration. Moreover, in the later stages of deficiency fluorescence is displayed throughout the skeletal musculature and in many other tissues of the body, which do not appear grossly abnormal in visible light. No yellow fluorescence was shown by the urinary bladder, brain, lungs or foetuses contained in a discoloured uterus. The yellow fluorescent substance has been concentrated by a process involving hydrolysis of the tissues with barium hydroxide, acidification, extraction with *iso*-butanol, transfer to dilute alkali, precipitation with acid and further purification by mild oxidation. The final product is a brownish yellow amorphous powder, which is soluble in butanol but almost insoluble in ether, chloroform or benzene. It has acidic properties, and in its fluorescence and solubility resembles the 'melanins' formed by the acid hydrolysis of proteins. A similar substance has been isolated from the brown atrophied heart of an aged woman. Further investigations of the properties of the fluorescent substance are in progress.

* Martin, A. J. P. & Moore, T. [1936]. *Chem. Ind.* **55**, 236; [1938] *ibid.* **57**, 973; [1939] *J. Hyg., Camb.*, **39**, 643.

Oxidizing Enzymes and Vitamin C in Tomatoes. By F. Wokes and J. G. Organ

The concentration of oxidizing enzymes is very high in the skin, fairly high in the flesh and very low in the juice of unripe and ripe tomatoes. The skin remains alkaline (*p*H 8·0–9·6) throughout ripening, but the *p*H of the flesh gradually falls to below 5 as ripening proceeds. The juice appears always to be acid (*p*H 4–5) in both unripe and ripe tomatoes. Destruction of vitamin C by ascorbic acid oxidase liberated from the tissues by grinding them with quartz sand is most rapid in the unripe skin (where 60–80 % may be lost in 1 min.), and rather less rapid in the flesh and juice of unripe green tomatoes. In ripe tomatoes the rate of destruction in the skin is almost as rapid as in unripe tomatoes, but much slower in the flesh and juice. In harmony with these findings, mincing of whole unripe tomatoes causes much more rapid loss of the vitamin than mincing of ripe tomatoes.* The concentration of vitamin C is about twice as high in the skin as in the flesh and juice. The concentration of the vitamin in smaller tomatoes, in which the skin forms a larger proportion of the total weight, may be a little higher, but the difference is not sufficient to discredit the findings of Sansome & Zilva† on polyploidy, as McHenry & Graham‡ had suggested.

* Wokes, F. & Organ, Joan G. [1942]. *Nature*, **150**, 523.
† Sansome, F. W. & Zilva, S. S. [1936]. *Biochem. J.* **30**, 54.
‡ McHenry, E. W. & Graham, M. [1935]. *Biochem. J.* **29**, 2013.

Vitamin B_1 and Toxaemia of Pregnancy. By R. KAPELLER-ADLER and J. A. CARTWRIGHT

Nineteen cases of pre-eclamptic toxaemia, eleven mild and eight severe ones, were treated with vitamin B_1 ('Benerva'). The treatment consisted usually of a course of ten intramuscular injections, 25 mg. of vitamin B_1 having been used on the first day and 10 mg. of vitamin B_1 on each of the following 9 days, but in some cases 10 mg. of vitamin B_1 were given orally instead of injections and a few patients received both.

In no case was there any beneficial effect which could be attributed to the vitamin B_1 and in some severe cases the vitamin seemed to accentuate the toxaemic symptoms.

The vitamin appeared to affect the histamine metabolism in these cases, diminishing the urinary excretion of histamine.

The Bearing of the Dissociation Constant of Urea on its Structure. By J. BELL, W. GILLESPIE and D. B. TAYLOR

The Chemical Findings in a Case of Acute Idiopathic Porphyrinuria. By J. E. KENCH

A case of this unusual condition was admitted to the Manchester Royal Infirmary under the care of Dr F. R. Ferguson, to whom I am indebted for the opportunity of making the investigations now reported.

The patient was a man of 27 whose symptoms were typical of porphyrinuria of the acute idiopathic type. For several years he had suffered from attacks of acute abdominal pain, accompanied by constipation, paralysis of the arms and bilateral wrist drop. Seventeen days after admission to hospital he had another periodic attack which proved terminal.

The urine was dark reddish brown, acid in reaction, and exhibited the two well-defined absorption bands at 580 and 542 mμ characteristic of the metaluroporphyrin complex excreted in this disease.* Acidification with hydrochloric acid caused the absorption bands to move to 600 and 557 mμ, whilst removal of the metal by treatment with sodium amalgam, followed by reoxidation, gave the free porphyrin spectrum. No protoporphyrin could be detected in the urine. The coproporphyrin values were 163 μg./litre before the attack and 128, 43 and 148 μg./litre during the attack, consisting of approximately equal amounts of isomers I and III.

After removal of ether-soluble porphyrins, uroporphyrin was separated by adsorption on Brockmann's alumina at *p*H 6·5, after boiling the urine for 5 min. at *p*H 3·7. The pigment was eluted with dilute ammonia, reprecipitated at *p*H 3·7, esterified in acid alcohol, and purified in the usual way. 191 mg. of uroporphyrin III methyl ester, m.p. 250°, mixed m.p. 249–257°, were obtained, representing 48 mg./litre of urine.

No uroporphyrin I was found, and these findings were confirmed by decarboxylation to the corresponding coproporphyrin III.

A specimen of stools collected in the remission period contained 0·13 mg. coproporphyrin per 100 g. fresh material (predominantly series III). The pigment excretion during the attack had risen to 1·81 mg./100 g. coproporphyrin. No uroporphyrin could be detected at any time.

The liver at autopsy exhibited diffuse passive congestion, the pigment content being 11·2 mg. coproporphyrin III and 50 μg. protoporphyrin (determined by ultraviolet fluorimeter).

No porphyrin could be detected in the blood either spectroscopically or by ultraviolet fluorescence, which may be considered evidence of the rapid and easy excretion of these pigments.†

* Rimington, C. [1938]. *Chem. & Ind.* **16**, 1110.

† Turner, W. J. [1938]. *Arch. Int. Med.* **61**, 762.

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PROCEEDINGS OF THE BIOCHEMICAL SOCIETY

The **226th Meeting** *of the Biochemical Society was held at the British Postgraduate Medical School, Ducane Road, Hammersmith, W. 12, on Saturday, 20 February* 1943, *at* 12 *noon, when the following papers were read:*

Vitamin B_1 in Malt Extract.

By F. Wokes and J. G. Organ

Vitamin B_1 in five different samples of malt extract was estimated by the thiochrome method using the technique of Nicholls, Booth, Kent-Jones, Amos & Ward,* and apparatus calibrated as previously described.† The results of over 200 consecutive assays, summarized in the accompanying table, when compared with microbiological results by Dr E. R. Dawson using the method of Schultz, Atkin & Frey,‡ led to the following conclusions:

(1) Quenching common in vitamin B_1 assays by the thiochrome method causes results to be too low. With malt extract the thiochrome results, if not corrected for quenching, may be less than a tenth of the microbiological values.

Series	Sample	No. of assays	Added aneurin as % of total aneurin	% of sample in solution used	Thiochrome results as per cent. of microbiological results calculating on % recovery of: Added aneurin — Mean	Added aneurin — S.D. of mean	Total aneurin — Mean	Total aneurin — S.D. of mean	Coefficient of variation for single assay based on % recovery of: Added aneurin	Total aneurin
					Unwashed					
1	A	16	21–35	5–40	61·9	± 9·9	82·3	±4·2	63·5	19·7
2	B	16	15–26	5–40	80·5	±13·9	87·3	±4·1	67·2	18·2
3	C	16	16–27	5–40	42·5	± 6·9	75·0	±5·4	64·4	28·0
				Mean	61·6		81·5		65·0	21·9
					Washed with isobutanol to remove quenching substances					
4	A	16	21–35	5–40	68·6	± 7·0	87·5	±1·9	27·9	8·5
5	B	16	15–26	5–40	63·3	±10·1	83·0	±3·0	61·6	14·3
6	B	16	15–26	5–40	100	±16·1	93·0	±1·9	63·0	8·1
7	C	16	16–27	5–40	59·4	± 6·0	92·0	±5·9	39·8	25·1
				Mean	72·8		88·9		48·1	14·0
					Washed and using high concentrations of added aneurin					
8	A	16	52–69	5–40	90·2	± 3·6	93·9	±2·2	14·5	8·7
9	B	16	42–59	5–40	97·0	± 8·8	95·0	±5·0	35·6	20·7
10	B	16	42–59	5–40	75·8	± 3·5	83·4	±1·9	17·9	9·1
11	C	16	42–59	5–40	89·7	± 5·0	94·9	±3·1	22·0	12·7
				Mean	88·2		91·8		22·5	12·8
					Fortified with extra aneurin in form of yeast					
Unwashed										
12	D	13	85–97*	5–20	96·0	± 5·5	99·0	±2·6	17·1	7·9
13	E	6	88–91	5–20	96·8	± 8·5	97·2	±3·5	21·5	8·9
					Weighted mean				18·5	8·2
Washed										
14	E	6	88–91*	5–20	98·0	± 4·9	98·5	±2·0	12·1	5·9

* Includes aneurin added as yeast and as pure aneurin. Standard deviations are for the mean result of all the assays in each series.

(2) Correction for quenching is usually based on the percentage recovery of *added* aneurin. This may deviate widely from the true percentage recovery (i.e. of *total* aneurin

calculated from microbiological results), causing the thiochrome results (*a*) to be much too low, (*b*) to have a very high coefficient of variation.

(3) Part of the quenching can be eliminated by washing the aqueous extract with isobutanol as recommended by Wang & Harris.§ This brings the results nearer to the microbiological values and also reduces the coefficient of variation, but the latter is still about six times the combined coefficients of variation of the thiochrome and microbiological methods as determined by replicate analyses.

(4) Flooding with aneurin (i.e. markedly increasing the proportion of added aneurin) combined with washing with isobutanol reduces the deviation from the microbiological values to a very low amount and the coefficient of variation to about twice the combined coefficients of the two methods.

(5) Since in over 200 consecutive assays the coefficient of variation was always greatly reduced by calculating results on recoveries of *total* instead of *added* aneurin the latter causes much of the experimental error of the thiochrome method. Low results on certain foodstuffs obtained by other workers using this method are probably largely due to inadequate compensation for quenching, which may help to explain deviations between thiochrome and biological results already observed by Harris & Wang.‖

(6) Comparison of aneurin content with diastatic power (old Lintner) gave: D.P. 12, 3·85 μg./g.; D.P. 112–132, 5·6–5·7 μg./g., in agreement with earlier findings of Bacharach & Allchorne ¶ that a higher D.P. was associated with higher content of vitamin B complex.

* Nicholls, J. R., Booth, R. G., Kent-Jones, D. W., Amos, A. J. & Ward, H. H. [1942]. *Analyst*, **67**, 15.
† Wokes, F. & Still, B. M. [1941]. *Chem. & Ind.* **60**, 722; Wokes, F., Organ, J. G. & Jacoby, F. C. [1942]. *Proc. Biochem. Soc.* **36**, vii.
‡ Schultz, A., Atkin, L. & Frey, C. N. [1942]. *Industr. Engng Chem.* (Anal. ed.), **14**, 35.
§ Wang, Y. L. & Harris, L. J. [1939]. *Biochem. J.* **33**, 1356.
‖ Harris, L. J. & Wang, Y. L. [1941]. *Biochem. J.* **35**, 1050.
¶ Bacharach, A. L. & Allchorne, E. [1928]. *Biochem. J.* **22**, 313.

The Vitamin P Activities of some British Fruits and Vegetables. By M. E. Coates and A. L. Bacharach

By the method previously described* biological tests have been carried out to compare the vitamin P activities of between 20 and 30 different vegetables or fruits and that of a provisional standard preparation, a water-soluble concentrate of citrus origin. Activities ranging from 0 (lentils) to 132 units (spinach) per 100 g. have been found. Full assays were carried out on 11 different vegetable products, which were contemporaneously tested for ascorbic acid content. There was no correlation. The vitamin P activity also bore little if any relationship to the colour of the product tested. The vitamin P content of peas was doubled after a few days' germination, but the ascorbic acid content increased nearly 20-fold.

* Bacharach, A. L., Coates, M. E. & Middleton, T. R. [1942]. *Biochem. J.* **36**, 407; Bacharach, A. L. & Coates, M. E. [1942]. *Analyst*, **67**, 313.

The State of Tyrosine in Egg Albumin and Insulin as shown by Spectrophotometric Titration. By J. L. Crammer and A. Neuberger

The change of the ultra-violet absorption spectrum of tyrosine with *p*H has been utilized to estimate the *pK* of the phenolic group in tyrosine, in diiodo-tyrosine and in proteins. The *pK* obtained for the two amino-acids agrees well with the values obtained by other methods. In insulin it can be shown that the phenolic groups are free, i.e. able to ionize,

and that the pK is displaced towards higher values. This is due to the negative charge of the molecules in alkaline solution. In negative egg albumin the phenolic groups cannot ionize; this is probably due to the fact that the phenolic groups are bound in a linkage, possibly a hydrogen bond. On denaturation this bond is broken and the phenolic groups ionize normally. It is believed that such linkages of phenolic groups are of major importance for the structures of native protein molecules.

An Examination of the Relationship between the Bacteriostatic Activity and the Oxidation-Reduction Potentials of Substituted Quinones. By J. E. PAGE and F. A. ROBINSON

Several workers have suggested that there might be a simple relationship between the oxidation-reduction potential of a substance and its antibacterial and related activities, and it was considered that the series of highly antibacterial quinones tested against strains of *Staph. aureus* and of *Bact. coli* under standard conditions by A. E. Oxford,* appeared to offer an opportunity of testing this theory.

We† examined the behaviour at the dropping mercury electrode of a number of mould metabolites and related quinones in acetate-buffered 75 % alcoholic solution and calculated the oxidation-reduction potentials (E_0') at pH 7·0 of these substances from their half-wave potentials. We failed to find a direct relationship connecting the reduction potential of these quinones with their bacteriostatic activity against strains of *Staph. aureus* and *Bact. coli*, but observed that, for *Staph. aureus*, the reduction potentials of all the active quinones examined to date fell between certain limits.

* Oxford, A. E. [1942]. *Chem. & Ind.* **61**, 189.
† Page, J. E. & Robinson, F. A. [1943]. *J. chem. Soc.* (in the Press).

A Rat Growth Method for the Assay of Pantothenic Acid. By J. S. D. BACON and G. N. JENKINS

A supplement to a basal diet and synthetic vitamins was sought which should provide the hitherto unidentified growth factors of the vitamin B complex but should contain no pantothenic acid. Acid and alkaline hydrolysates of liver extracts did not fulfil the above condition, as the pantothenic acid was not destroyed completely. Wheat germ, however, fed at a level of 0·5 g. per day fulfilled these conditions approximately. A satisfactory curve was constructed relating growth of rats to dosage of pantothenic acid over a range of 0–100μ daily given to rats receiving basal diet, synthetic B vitamins and wheat germ.

This result provides a rat growth method for assaying pantothenic acid in foods.

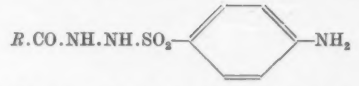
Propylene Glycol as a Solvent for the Intravenous Injection of DOCA. By E. P. SHARPEY-SCHAFER

'March' Haemoglobinuria in a Soldier. By H. S. MITCHELL and R. A. PALMER

Phenolphthalein Phosphate as a Reagent for Studying the Phosphatase Content of Organisms. By J. BRAY and E. J. KING

Concentration of Sulphanilamide Derivatives in Bile of Rabbits. By MARY BARBER, J. H. DIBLE and G. A. D. HASLEWOOD

A compound of cholic acid and sulphanilamide (sulphacholazine) having the formula



(R = cholyl) was prepared by one of us* in the hope that it might be concentrated in the bile. This compound has considerable bacteriostatic activity *in vitro* against *Strep. haemolyticus* and *Strep. pneumoniae*, but little or none against members of the coliform group of organisms.

A series of rabbits have been injected intravenously with sulphacholazine, sulphanilamide and sulphapyridine respectively. Attempts have been made to estimate the concentration of each substance reached in the bile by means of the diazo reaction after first precipitating the bile with alcohol. This reaction produced no colour with bile from normal rabbits, and the red colour which developed in the bile of the experimental animals was compared in a colorimeter with that of a known standard of the compound originally injected. These experiments indicate that sulphacholazine reaches a high concentration in the bile of rabbits, whereas sulphanilamide and sulphapyridine are never present in the bile except in very small amounts. Attempts to demonstrate that bile containing high concentrations of sulphacholazine is more bactericidal *in vitro* than normal rabbit bile have so far failed, owing to the highly strepticidal nature of the latter.

* Haslewood, G. A. D. [1941]. *Biochem. J.* **35**, 1307.

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PROCEEDINGS OF THE BIOCHEMICAL SOCIETY

The **Annual General Meeting** *of the Biochemical Society was held at the Courtauld Institute of Biochemistry, Middlesex Hospital, London, W. 1, on Saturday, 27 March* 1943, *at 2 p.m., when the following papers were read:*

The Measurement of Fluorescence Intensity. By P. Ellinger and M. Holden

Examination of physical conditions governing measurement of fluorescence intensity carried out to develop a fluorimeter suitable for routine purposes reveals that the photoelectric cells are most satisfactory analysers. Relative arrangement of analyser, container of fluorescent solution and light source should be in straight line so that light intensity measured is dependent exclusively on concentration of fluorescent material. Primary light can be excluded from analyser by suitable filters and should be monochromatic to obtain absorption corresponding to Beer's law. 365 mμ mercury line is suitable light source. Interference with measurement caused by fluorescence maximum, mainly dependent on absorption, can be avoided by using narrow containers and high primary light intensity. Measurement can be made either with one photocell by measuring galvanometer deflexion and relating it to primary light intensity or by compensating methods involving two photocells and using the galvanometer as a null instrument, balancing either the light by altering the aperture of primary or secondary beam, or the photocell currents with the potentiometer. Comparative results (table) show potentiometer method superior in range, speed and accuracy. A fluorimeter designed on these principles is described.

Comparative results obtained on quinine sulphate with various fluorimeter arrangements

		Balancing methods		
		Altering the aperture of		
	Direct deflexion measurement	Primary beam	Secondary beam	Potentiometer
Range	1 : 500	1 : 32	1 : 16	1 : 128
Speed (time for 10 readings)	24·5′	14·5′	15′	9′
Standard deviation in % of mean (10 readings)	1·2–5·0	0·17–1·5	0·54–1·4	0·06–2·3

A Chromatographic Separation of Coproporphyrin from Uroporphyrin; an Improved Method for the Preparation of Uroporphyrin from Pathological Urines. By C. Rimington

In urines from cases of acute porphyry, the porphyrins are accompanied by large quantities of dark brown or red pigments which are very difficult to remove. All adsorbents so far recommended are used to adsorb the porphyrins in the region of their isoelectric point, at which reaction the accompanying pigments are also retained. Attempted precipitation of the crude porphyrin by means of acetic acid is not always successful and any precipitate obtained contains a mixture of coproporphyrin and uroporphyrin which is troublesome to separate.

Kieselguhr possesses the remarkable and very desirable property of adsorbing both coproporphyrins and uroporphyrins from solution *in dilute hydrochloric acid* whilst rejecting almost entirely the dark brown, non-porphyrin pigments. Elution by buffer solutions may be made selective by taking advantage of the fact that uroporphyrins I and III are removed at *p*H 5·25, while coproporphyrins I and III, no doubt by virtue of their higher isoelectric point, are not eluted until the *p*H of the buffer reaches 5·5 or 6·0. It has not been possible to separate the individual isomers of each porphyrin from each other by this means.

As a preparative procedure the method was applied to a very dark specimen of urine from a case of acute porphyry as follows: To the urine, hydrochloric acid was added to a concentration of 1 % and the mixture passed through a Tswett column of acid-washed kieselguhr (B.D.H.). The deeply coloured filtrate was free from porphyrin. The column was now washed successively with 1 % HCl, glacial acetic acid (which removed a little more brown pigment and a small quantity of porphyrin) and water. Molar acetate buffer *p*H 5·25 was now used and removed the uroporphyrin as a deep red solution substantially free from other pigment. Upon acidification, the porphyrin precipitated readily and was removed for esterification in the usual way.

A Method for the Quantitative Determination of Uroporphyrin in Pathological Urines. By C. Rimington

Great difficulties attend the quantitative determination of uroporphyrin. Adsorption methods are usually attended by loss and do not remove accompanying dark pigments. Uroporphyrin III is sparingly soluble in ethyl acetate under strictly controlled conditions of *p*H, but uroporphyrin I is not extractable from urine by this or any other known solvent immiscible with water.

A satisfactory quantitative procedure has been elaborated based upon the selective adsorption of the porphyrins from acid solutions (1 % HCl) by kieselguhr.

In order to convert all porphobilinogen or other precursors into porphyrin, hydrochloric acid is added to the urine to a concentration of 1 % and the mixture left overnight or, less desirably, heated for 1 hr. in the water-bath. An aliquot (usually 1–3 ml.) containing 2–20 μg. of porphyrin is diluted in a centrifuge tube with 1 % HCl to about 5 ml., 0·7 g. acid-washed kieselguhr (B.D.H.) added and after vigorous shaking the mixture centrifuged. The adsorbent is washed by shaking successively with two lots of 1 % HCl and one of water. The porphyrin is now eluted by stirring with three successive lots of about 7 ml. each of 0·1 *N* NaOH and the combined eluate made up to 25 ml. Small particles of kieselguhr may render this alkaline solution turbid, but if left overnight they sink, leaving a perfectly clear and almost colourless supernatant, a portion of which is suitably diluted with 0·1 *N* NaOH and the porphyrin content determined in a fluorescence comparator (optimal porphyrin concentration about 0·25 μg./ml.). This measures the combined uroporphyrin and coproporphyrin. Coproporphyrin is determined separately by the ether extraction method, either in the eluate or in the original urine, and the uroporphyrin calculated by difference. As an illustration of the method, the daily output of uroporphyrin and coproporphyrin by a case of acute porphyrinuria is presented. The period of 23 days includes an attack and remission.

The Toxicity of Pure Vitamin A. By T. Moore and Y. L. Wang

It has long been known that greatly excessive doses of vitamin A concentrate are poisonous to rats, but opinions have been divided as to whether the toxicity is due to vitamin A itself or to some accompanying impurity. Tests of the toxicity of crystalline vitamin A

acetate ($E^{1\%}_{1\,cm.}$ = 1500 for acetate) were made possible through the kindness of British Drug Houses Ltd., in providing a generous sample. Doses of about 25,000–50,000 i.u. daily to young rats of 69–79 g. body weight invariably caused fractures of the bones after periods of 8–20 days. Doses of about 50,000 given to adult pregnant rats did not cause breakages of the bones within the period of the experiment, but gave rise to fatal uterine haemorrhage, as originally described by Hartwell in rats given excess of cod-liver oil. This phenomenon appears to be one manifestation of the tendency to haemorrhage in hypervitaminosis A. Other forms of haemorrhage are subcutaneous or intramuscular haemorrhage which occurs frequently in the legs; massive and rapidly fatal haemorrhage under internal membranes, such as the pericardium; or excessive haemorrhage as the result of minor external injury. In some respects the tendency to haemorrhage in hypervitaminosis A has a strong superficial resemblance to that found in scurvy.

The Metabolism of Azo Compounds. By L. A. Elson and F. L. Warren

Crystallization on Foam. By R. Bader and F. Schütz (introduced by W. V. Thorpe)

When solutions of bile salts are partly converted into foam, the acids crystallize in different fractions of the foam. Crystals of bile acids can readily be obtained from crude commercial specimens of bile salts. Some conditions of crystallization and a number of principles of the foam method are discussed, which have been derived from measurements of foam stability* of the substances involved.

* Schütz [1937]. *Nature, Lond.*, **139**, 629; [1938], *C.R. Soc. Physiol. Suisse*, **46**; [1942], *Trans. Faraday Soc.* **38**, 85, 94.

Correlation of the Oxygen Capacity, Iron Content and Absorption Spectrum of Haemoglobin of Normal Subjects. By H. M. Jope, E. M. Jope and J. R. O'Brien

It has become increasingly evident that the word 'haemoglobin' as applied to whole blood could not be adequately defined. We have accordingly set out to determine the extent of correlation of its chief properties—O_2 and CO combining power, iron content and absorption spectrum—as found in whole blood, and if possible to relate our results to the properties of some definable solid haemoglobin preparation. The results from the first 25 normal human bloods examined are reported here.

	Fe : O_2 c.c. O_2/1 g. Fe	Fe : Extinct. coeff. Soret band $E^{1\ g.\ atom\ Fe/l.}_{1\ cm.} \times 10^{-3}$	O_2 : Extinct. Soret band $E^{1\ mol.\ O_2/l.}_{1\ cm.} \times 10^{-3}$
Theoretical			
1 atom Fe : 1 mol. O_2	401	—	—
Our mean	396	132	138
Extreme range	367–418	127–141	125–152
Standard deviation, as % of mean	3·3%	3·2%	5%

The relation of absorption spectra of carboxy- to oxyhaemoglobin appears to be even more irregular. All the experimental methods used are reproducible to within ±0·2–0·5%. The discrepancies in the exact correlation would therefore seem to be very largely real: it is unlikely that they are due to systematic errors in the methods. Our results indicate that it is not adequate to define the absolute value of one property in terms of another, and hence the standard for, say, a photometric method in terms of a gasometric method.

The possible uncertainty of defining the water content of crystalline preparations of proteins made it desirable to relate these data to a preparation of haemoglobin dried under standard conditions. The procedure of Morrison & Hisey,* with a few modifications, gave dried horse haemoglobin, which redissolved easily in water. This clear solution carried 409 c.c. O_2/g. Fe, which indicates that every iron atom present was capable of carrying oxygen.

* Morrison, D. B. & Hisey, A. [1935]. *J. biol. Chem.* **109**, 23.

Further Observations on the Excretion of Coproporphyrin in Normal Subjects.
By E. M. Jope and J. R. O'Brien

1. *Definition of standard.* From a study of synthetic and natural coproporphyrins I and III the absolute extinction coefficients for the free porphyrins in 0·5 % HCl are defined:

$$548\ \mathrm{m}\mu = E\,{}^{1\%}_{1\,\mathrm{cm.}} = 260, \quad 401\ \mathrm{m}\mu = E\,{}^{1\%}_{1\,\mathrm{cm.}} = 8100.$$

III is equivalent to I within ± 1 %.

2. *Normal range of coproporphyrin excretion.* (*a*) *Urine*: in 3-day collections, below 100 μg./day, though isolated 24 hr. values may be 100–150 μg./day. The lower limit so far observed is 10 μg./day. (*b*) *Faeces*: 3-day averages are 150–350 μg./day: shorter collection periods give results too erratic to be usable. Any one individual varies less than this overall normal range.

3. The excretion of coproporphyrin in urine throughout the 24 hr. is irregular: estimations on single urine specimens are therefore of little value.

4. Maechling* reports increased coproporphyrin in pathological urines after standing 2 hr. with $\frac{1}{10}$ vol. conc. HCl. We have confirmed this occasionally in pathological cases but have not observed any increase in normals.

5. Evidence in the literature suggests that the normally excreted coproporphyrin is largely the I isomer, but that some III may occur. I is more easily recognized and Fischer originally missed the III in the famous porphyria case Petry.†

A knowledge of the I : III ratio normally excreted is now most imperative for the interpretation of the evidence accumulating from pathological conditions. Subjects should be studied separately, as pooling of extracts may lead to a result of doubtful significance.‡ We have found a I : III ratio of 4·3 : 1 in one normal urine and both faeces and urine of others are under examination.

* Maechling, E. H. [1940–1]. *J. Lab. Clin. Med.* **26**, 1676.

† Mertens, E. [1939]. *Hoppe-Seyl. Z.* **258**, Suppl. 1.

‡ E.g. Grotepass, W. [1938], *Hoppe-Seyl. Z.* **253**, 276, who used 10,000 l. of pooled hospital urine and obtained an equivocal result.

[*The Editors of the Biochemical Journal accept no responsibility for the Reports of the Proceedings of the Society. Abstracts of papers read are published as received from the author.*]

PROCEEDINGS OF THE BIOCHEMICAL SOCIETY

The **228th Meeting** *of the Biochemical Society was held in the Department of Medical Chemistry, the University, Edinburgh, on Saturday, 5 June* 1943, *at* 2.30 *p.m., when the following papers were read:*

Heated Cholesterol as a Potential Carcinogen. By A. H. M. KIRBY

The high proportion of human cancer deaths due to stomach cancer and the extreme rarity of animal stomach cancer suggest a carcinogen in human food. Roffo* claimed adenocarcinoma in rats fed fats or cholesterol heated ½-hour to 350° C. Beck & Peacock† were unable to confirm for heated fats but found papillomatosis of forestomach due to induced avitaminosis-A.

Groups of Wistar rats (on basal diet of rat-cake,‡ water *ad lib.*, milk and greenstuff) were fed:

(1) Unheated cholesterol: 20 mg. each/day (controls).

(2) Cholesterol heated to 270–300° C. for ½-hour (unchanged sterol removed): 20 mg. each/day.

(3) Residue from (2) after cholesterone and dicholesteryl ether removed: 20 mg. each/day.

(4) $\Delta_{3,5}$ Cholestadiene (claimed carcinogenic by Waterman§): 25 mg. each/day.

Only hyperkeratosis and 1 papilloma seen in two years. No tumour seen in other rats fed heated cholesterol esters or cholesterol heated to 430° C. (fifteen months).

Solutions of above products in olive or arachis oil injected subcutaneously into mice by Dr S. Beck; only two fibrosarcomata seen in total of 90 mice (Steiner, Steele & Koch¶).

Roffo's claim that heated cholesterol contains a polycyclic hydrocarbon very similar to 3:4-benzpyrene not confirmed. Substance with strong blue fluorescence in U.-V. beam present, but absorption maxima lie at 2330; 2440; 2560 and 2615 Å.

* Roffo, A. H. [1939]. *Bull. Ass. franç. Cancer*, **28**, 556; *Z. Krebsforsch.* **49**, 341.
† Beck, S. & Peacock, P. R. [1941]. *Brit. Med. J.* (ii), 81.
‡ Thomson, W. [1936]. *J. Hyg., Camb.*, **36**, 24.
§ Waterman, N. [1939]. *Acta Union Int. contre Cancer*, **4**, 764.
¶ Steiner, P. E., Steele, R. & Koch, F. C. [1943]. *Cancer Res.* **3**, 100.

The State of Vitamin A and Carotene in Human Blood Plasma. By DZIAOSZYNSKI, E. M. MYSTKOWSKI and C. P. STEWART

The Ribonucleic Acid Content of Mammalian Tissues. By J. N. DAVIDSON and C. WAYMOUTH

It is now recognized that ribonucleic acids (yeast nucleic acid, phytonucleic acid) are not found exclusively in yeast cells and plant tissues but occur also in animal tissues, e.g. pancreas, along with desoxyribonucleic acid (thymonucleic acid, animal nucleic acid).

Using chiefly material from the adult and embryo sheep we have demonstrated the presence of ribonucleic acids in all tissues so far examined. The nucleic acids were extracted from the tissues by means of 10% sodium chloride and were precipitated as lanthanum salts. Ribose and desoxyribose were colorimetrically estimated in the precipitate with the aid of the Spekker Absorbtiometer using calibration curves drawn

up in terms of ribonucleic acid phosphorus (RNAP) and desoxyribonucleic acid phosphorus (DNAP). The presence of ribonucleic acid was confirmed enzymatically with the aid of crystalline ribonuclease* which attacks specifically ribonucleic acids.

Although in most tissues the amount of total nucleic acid was much higher in the embryo than in the adult, the ratio of RNAP to DNAP was either of the same order in the embryo as in the adult or was somewhat higher in the latter. The ratio was high in such tissues as brain, heart, and liver and low in spleen and thymus.

Some of the results obtained support the view that ribonucleic acid preponderates in the cytoplasm and desoxyribonucleic acid in the nuclei.

* Kunitz, M. [1940]. *J. Gen. Physiol.* **24**, 15.

The Response of Protein Metabolism to Injury. By H. N. Munro and D. P. Cuthbertson

Both in the human being and in the rat, injury such as the fracture of a long bone causes a marked rise in the excretion of N, P, K and creatine which is maximal about the third day after injury (Cuthbertson, M'Girr & Robertson*). As this excessive excretion of N could not be accounted for by loss of tissue substance locally at the site of fracture, it was concluded that a generalized tissue breakdown took place to meet the emergency.

Pursuing the origin of this N further, we have studied the response to fracture of rats when they are fed on a protein-free but otherwise qualitatively adequate diet slightly deficient in energy value. Two sets of 4 rats each were fed on a protein-free diet until the N excretion fell to a steady level; fracture of the left femur was then carried out and their urinary N output followed on the same dietary regime for a further 8 days. No rise in N output was found in either group. An identical operative procedure on animals fed at two different levels of protein intake gave metabolic responses similar to those reported in previous publications.

We are therefore persuaded that the excessive output of N which follows injury arises from storage-protein and not from essential tissue substance.

* Cuthbertson, D. P., M'Girr, J. L. & Robertson, J. S. M. [1939]. *Quart. J. Med.* **29**, 13.

Investigations on the Activity of Histaminase in Normal and Toxaemic Pregnancy. By R. Kapeller-Adler

An Inexpensive Balanced Photoelectric Colorimeter for Mains Operation. By G. H. Bell and A. R. Smellie (introduced by G. M. Wishart)

The photoelectric haemoglobinometer shown by Bell & Guthmann* to the Society last year has proved to be very satisfactory; the lamp cannot, however, be supplied from the mains. In the present form two photocells and two light filters are required; the photocells are placed equidistant from a single light and are connected in parallel opposition directly to the galvanometer. With water in the glass cells in front of the photocells the area of illumination of the reference photocell is altered by a shutter until the galvanometer is brought to zero. The circuit is now balanced for quite considerable light variations; the lamp can be supplied from A.C. or D.C. mains. The rest of the procedure, including the calibration of the E scale, is as previously described.

* Bell, G. H. & Guthmann, E. [1942]. *Biochem. J.* **36**, *Proc.* 6 June 1942, iii.

The Vitamin P Potency of Foodstuffs. By H. SCARBOROUGH

By determinations of alterations in capillary resistance in man, an attempt has been made to assess the vitamin P potency of certain fruits and vegetables. 'Good' sources of the vitamin include grapes and grape juice, both dark and light varieties, lemons and orange juice, pulp and peel, plums, especially the darker coloured varieties, prunes, 'citrin' prepared according to the method of Szent-Györgyi (supplied by Roche Products Ltd.), a water-soluble concentrate prepared from blackcurrants by Dr A. Pollard, Long Ashton Research Station (W.S.P. 4, Bacharach & Coates*) and preparations of rose hips (supplied by Dr F. Wokes, Ovaltine Research Laboratories). 'Moderately good' sources include grape-fruit juice and pulp, dried apricots, cherries, blackberries and rose-hip purée (prepared by M. Andross†). 'Poor' sources include most green vegetables, tomatoes, potatoes and a blackcurrant purée (supplied by M. Oliver, Chivers Ltd.).

On the basis of the data obtained by Bacharach & Coates with guinea-pigs, 'good' sources contain more than 100 'provisional units' per 100 g.; 'moderate' sources contain approximately 100 units per 100 g.; 'poor' sources contain under 100 units per 100 g.

* Bacharach, A. L. & Coates, M. E. [1942]. *Analyst*, **67**, 313.
† Andross, M. [1941]. *Analyst*, **66**, 358.

The **229th Meeting** *of the Biochemical Society was held in the Department of Biochemistry, University of Sheffield, on Saturday,* 10 *July* 1943, *at* 2.30 *p.m., when the following papers were read:*

Pantoyltaurine-resistance in Bacteria; the Experimental Derivation of Drug-resistant Strains in the Absence of any Inhibitor. By H. MCILWAIN

Different strains of *Corynebacterium diphtheriae* showed varying degrees of resistance to pantoyltaurine, which was partly correlated with their ability to grow with β-alanine in place of pantothenate; sensitive strains required added pantothenate. The correlation was first found in naturally occurring strains but extended to those which were made resistant to the drug by growth in its presence. Typical pantoyltaurine-fast strains synthesized pantothenate, and were also prepared by training strains to grow without added pantothenate. Pantothenate synthesis is thus a major factor in their pantoyltaurine resistance.

Natural strains of group A haemolytic streptococci were uniformly sensitive to pantoyltaurine, but sensitive organisms of this and other groups were made resistant to the drug by suitably arranged growth in its presence. Resistance in these and in some other streptococcal strains was not by pantothenate synthesis. Some organisms insensitive to pantoyltaurine alone were made sensitive to it by the presence of salicylate in sub-inhibitory concentrations. Such synergism was specific to salicylate. The joint inhibition was antagonized by pantothenate, which also antagonized inhibitions due to limiting concentrations of salicylate alone. These organisms, also, appear to owe their resistance to a modification of processes which involve pantothenate.

Partition Chromatography of Free Amino-acids and Peptides. By A. H. GORDON, A. J. P. MARTIN and R. L. M. SYNGE

The data of England & Cohn* on the distribution of amino-acids between BuOH-H_2O phases suggest that this system, originally employed for analytical purposes by Dakin, should give good separations of amino-acids when used in a partition chromatogram.

When silica gel is employed to hold the aqueous phase, poor resolutions are obtained, presumably owing to adsorption by the silica of the substances which it is desired to separate. By substitution of cellulose for silica more promising results have been obtained.

* England & Cohn [1935]. *J. Amer. chem. Soc.* **57**, 634.

An Oxidase System in the Potato Tuber. By J. G. Boswell

The Protection of Tocopherol during Saponification with Pyrogallol. By T. Moore and J. Tošić

The instability of tocopherol to alkali presents a serious difficulty in the estimation of the vitamin by the method of Emmerie & Engel.* During the usual procedure of rapid saponification of the fatty medium with hot alcoholic potash much vitamin may be lost. On the other hand, the reaction of Emmerie & Engel is not given by tocopherol acetate, which suggests that vitamin present in esterified forms in natural sources might escape estimation if saponification were to be omitted. The destruction of the vitamin presumably depends on the presence of oxygen, but Lester Smith & Bailey† found that rapid saponification in an atmosphere of inert gas gave no higher results than their ordinary procedure, in which 87 % of synthetic α-tocopherol added to wheat-germ oil was recovered.

We have found by means of recovery tests that loss may be completely prevented by the convenient procedure of adding pyrogallol before saponification. The pyrogallol is removed during the usual washing of the ether layer (with 0·5 % potash and water) before evaporating. The following results have been obtained:

Recovery of α-tocopherol during saponification in presence and absence of pyrogallol

α-Tocopherol mg. added	Without pyrogallol		With pyrogallol	
	mg. found	% recovery	mg. found	% recovery
	α-Tocopherol acetate in absence of oil			
1·78	0·66	37·4	1·76	98·8
1·78	1·04	58·5	1·77	99·4
	α-Tocopherol acetate dissolved in arachis oil			
2·06*	1·84	89·3	2·02	98·2
2·06	1·80	87·4	2·03	98·6

* Vitamin E present in the oil is made up as follows: 1·82 mg. in the form of added synthetic α-tocopherol acetate + 0·24 mg. found in the arachis oil itself by our pyrogallol method.

The much smaller loss of tocopherol when dissolved in arachis oil may be due to the presence in the fat of substances capable at least of reducing the amount of oxygen available for the destruction of the vitamin. Experience with numerous vegetable fats has shown that the pyrogallol procedure gives consistent results. Without pyrogallol substantially lower values are obtained, the extent of the deficit depending on the nature of the oil.

The value of pyrogallol for the protection of vitamin E during the digestion of tissues and foodstuffs, with alkali, and for the preservation of other unstable substances under similar circumstances is under investigation.

* Emmerie, A. & Engel, C. [1938]. *Rec. Trav. chim.* **57**, 1351.

† Lester Smith, E. & Bailey, R. [1939]. Vitamin E. A symposium, p. 21. Cambridge: Heffer.

The Effect of Glycocyamine on the Formation of Tumours induced by Acetyl-amino-fluorene. By F. BIELSCHOWSKY (introduced by H. A. KREBS)

2-Acetyl-amino-fluorene is a carcinogenic agent for the white rat.* 4 mg. of this compound (per rat and day), added to the standard diet for 20–30 weeks, produce malignant tumours in different organs. In female rats the majority of these tumours are adenocarcinomas of the breast which become evident on average after 250 days.

The addition of 20 mg. of glycocyamine (per rat and day) to the acetyl-amino-fluorene containing diet accelerates the appearance of breast tumours and at the same time increases considerably the number of liver tumours in female rats.

These results suggest that the reduction of available labile methyl-groups brought about by the conversion of glycocyamine into creatine enhances the carcinogenic action of 2-acetyl-amino-fluorene.

* Wilson, R. H., DeEds, F. & Cox, A. J. [1941]. *Cancer Res.* **1**, 595.

Determination of Isocitric Acid in Biological Material. By H. A. KREBS and L. V. EGGLESTON

The neutral solution of (−)isocitric acid is incubated with aconitase and the citric acid formed is estimated. Aconitase, unlike the related enzyme fumarase, is very labile in aqueous tissue extracts, but the enzyme is stable for several weeks in frozen minced pigeon breast muscle stored at −1°. Citric acid is determined by a modification of the volumetric method of Pucher, Vickery & Leavenworth.* For the calculation of the isocitric-acid concentration it is necessary to know the composition of the equilibrium mixture of citric, isocitric and *cis*-aconitic acids in the presence of aconitase. It was found to contain 89·5% citric, 6·2% isocitric and 4·3% aconitic acid. The higher estimate of isocitric acid of earlier authors, based on polarimetric measurements, arose from the assumption that $[\alpha]_D$ for the molybdate complex of isocitric acid was, under the test conditions, −428°. Citrate, however, increases $[\alpha]_D$ of the complex to −780°. If a is the amount of citric acid in the sample before, and b the amount after, treatment with aconitase the quantity of isocitric acid in the sample is $1{\cdot}117 \times b - a$. The occurrence of isocitric acid in various plants† is confirmed.

* Pucher, G. W., Vickery, H. B. & Leavenworth, C. S. [1924]. *Industr. Engng Chem.* (Anal. ed.), **6**, 190.
† Pucher, G. W. & Vickery, H. B. [1942]. *J. biol. Chem.* **145**, 525.

The Oxidation of Acetic Acid in Acetobacter. By J. TOŠIĆ and H. A. KREBS

Acetic acid is an end-product in *Acetobacter* when ethanol is present as a substrate, but is readily oxidized under other conditions. This observation was investigated in detail as it was thought that it might shed light on the mechanism of the biological oxidation of acetic acid. Washed suspensions of *Acetobacter turbidans* were used for most experiments. Suspensions of the organism do not absorb oxygen immediately on addition of acetate, but acquire the ability to oxidize acetate during a lag period, the duration of which increases on storage. The lag period is abolished or reduced, and the rate of oxidation of acetate is increased, by the addition of small quantities of pyruvate or some other oxidizable substances (lactate, glucose, malate, α-ketoglutarate); ethanol and propanol have no effect. In order to test whether the action of the various substrates on the oxidation of acetate is due to the synthesis of a specific catalyst during the oxidation of the substrates the effect of the B vitamins and other compounds on the lag period was tested. None of the vitamins was able to replace the oxidizable substrate. Adenyl-pyrophosphate shortened the lag period and increased the rate of acetate oxidation, but the effect was somewhat smaller than that of pyruvate.

The **230th Meeting** *of the Biochemical Society was held at St Thomas's Hospital Medical School, Manor House, Godalming, Surrey, on Saturday, 25 September* 1943, *at* 12 *noon, when the following papers were read:*

Further Observations on the *C. diphtheriae* Growth Factor of the B Complex. By F. W. Chattaway, F. C. Happold and Mary Sandford

The paper constitutes a further report on the properties of the *C. diphtheriae* growth factor present in the amyl-alcohol insoluble residues of extracts of whole liver.

The factor is relatively stable to acid, and is markedly labile to alkali, nitrous acid, ninhydrin and hydrogen peroxide. Methylation and acetylation are highly destructive and activity cannot be regained by acid hydrolysis. Adsorption occurs on norite charcoal but not on Fuller's earth. Extraction cannot be effected by diethyl ether, chloroform, petroleum ether, amyl alcohol, butyl alcohol or *p*-cresol at determined *p*H values. Precipitation of the active component does not occur with silver salts at acid and neutral reactions, and occurs only slightly with silver sulphate in the presence of baryta at *p*H values above 8·0.

Lead acetate is ineffective as a precipitating agent at *p*H values of 2·8, 6·9 and 9 to 10.

Phosphotungstic acid in 5% sulphuric acid removes inert material from solution.

The activity of the most purified preparations is such that the addition of 5γ dried material per ml. of basal medium promotes moderate growth whereas one-hundredth of this amount evokes a detectable growth response. It is free from all traces of *L. casei* ϵ growth factor.

A Tissue Growth-Promoting Factor present in Pancreatin. By J. N. Davidson and C. Waymouth

Commercial pancreatin contains a factor which stimulates the growth in roller tubes by Willmer's* technique of fresh explants of the 9-day chick embryo heart.† When the factor is added along with embryo extract to roller tubes containing six fresh explants (embedded in fowl plasma) which have been cultivated in Tyrode solution for 48 hr., it produces in the course of a further 48 hr. a much greater rise in nucleoprotein phosphorus (N.P.P.) in the cultures than does embryo extract alone.

Composition of fluid phase in roller tubes in second 48 hr. of test	Change in N.P.P. μg. per roller tube
Embryo extract alone	+0·36
Embryo extract + crude factor	+0·82
Embryo extract + factor heated at 100° for 30 min. at *p*H 10	+0·39
Tyrode solution alone	−0·09
Purified factor (N = 50 mg./100 ml.) in Tyrode *without* embryo extract	+0·66

The factor is stable to heat in neutral or slightly acid solution, but is inactivated by heating in faintly alkaline solution. It can be purified by extraction with phenol and the purified material exerts a powerful growth-promoting action on chick heart fibroblasts even in the absence of embryo extract.

* Willmer, E. N. [1942]. *J. exp. Biol.* **19**, 11.

† Davidson, J. N. & Waymouth, C. [1943]. *Biochem. J.* **37**, 271.

Studies on the Plasma Fibrinogen and other Protein Fractions in a Case of Afibrinogenaemia. By J. L. Pinniger and F. T. G. Prunty

By adaptation of the Kark and Soutar* prothrombin technique, and from precipitin studies, evidence is presented for complete lack of fibrinogen in a girl with haemorrhagic

symptoms. The relation of the *in vitro*† and *in vivo* fibrinogen to the formation of clots, and the effects of transfusion of concentrated plasma on the protein fractions of the patient's plasma have been investigated.

* Kark & Soutar [1940]. *Lancet*, **1**, 1149.
† Witts, L. J. [1942]. *J. Path. Bact.* **54**, 516.

Fluorescent Derivatives of N-Methyl-Chloro-Nicotinamide. By R. A. COULSON and P. ELLINGER

The supposition that F_2 might be thiochrome* proved to be incorrect.† The analogy with the elimination of methylated products after the intake of pyridine‡ and nicotinic acid§ suggested the possibility of the formation of methyl derivatives of nicotinamide which might be related to F_2.†‖ The natural non-fluorescent precursor of F_2 was crystallized from KCl eluates of urine rich in the compound by low temperature vacuum distillation, fractional precipitation of KCl and other impurities, and by adsorption on Al_2O_3 and elution with methanol. An orange aurate (m.p. 164°), a yellow mercury-potassium iodide (m.p. 136·3°) and a yellow picrate (m.p. 190·2°) were formed from the concentrate. The melting-points of the latter two were identical to the corresponding salts of synthetic N-methyl-chloro-nicotinamide†¶ and the mixed melting-points showed no depression. The aurate differed in melting-point and colour (synthetic: 170·2° yellow) (possibly two aurates as in trigonelline).**

The transformation by alkali of N-methyl-chloro-nicotinamide into two fluorescent compounds F_{2a} and F_{2b}† is nearly complete in dry isobutanol, whereas in water it is incomplete and the destruction is rapid (cf. formation of a carbinol from N-methyl-pyridium hydroxide††). The maximum yield by extraction of the alkaline aqueous phase by isobutanol is only 20 %. The predominance of the quaternary ammonium compound in water and of the carbinol† in isobutanol is evident from the spectrographs (prepared by Dr E. M. F. Roe), the shift of which resembles that obtained for the non-reduced and reduced N-methyl-iodo-nicotinamide‡‡ respectively.

* Coulson, R.A., Ellinger, P. & Platt, B. S. [1942]. *Biochem. J.* **36**, xii.
† Ellinger, P. & Coulson, R.A. [1943]. *Nature, Lond.* (in the Press).
‡ His, W. [1887]. *Arch. exp. Path. Pharmak.* **22**, 253. Cohn, R. [1893]. *Hoppe-Seyl. Z.* **18**, 16; and many others.
§ Ackermann, D. [1913]. *Z. Biol.* **59**, 18. Linneweh, W. & Reinwein, H. [1932]. *Hoppe-Zeyl. Z.* **207**, 48; **209**, 110.
‖ Najjar, V. A., Scott, D. B. M. & Holt, jr., L.E. [1943]. *Science*, **97**, 537. Huff, J. W. & Perlzweig, W. A. [1943]. *Science*, **97**, 538.
¶ Karrer, P., Schwarzenbach, G., Benz, F. & Sollmsen, V. [1936]. *Helv. Chim. Acta*, **19**, 826.
** Jahns, E. [1885]. *Ber. dtsch. chem. Ges.* **18**, 2518; [1887] **20**, 2840.
†† Hantzsch, A. & Kalb [1899]. *Ber. dtsch. chem. Ges.* **32**, 3117; Decker, E. & Kaufmann, A. [1911]. *J. prakt. Chem.* [2] **84**, 432.
‡‡ Warburg, O. & Christian, W. [1936]. *Biochem. Z.* **287**, 315.

The Excretion of Histidine in Urine. By J. R. O'BRIEN and P. E. QUELCH

Studies in the metabolism of histidine* in pregnancy have raised problems, the solution of which would be aided by a sound method of estimation. One group of methods uses Knoop's reaction for this amino-acid which, although almost specific for histidine,† is sensitive to urinary constituents such as uric acid, phosphate and urea. To eliminate interfering substances a method based upon a base-exchange between histidine and permutite was worked out for urine. Acidified urine is allowed to percolate through a column of permutite, which is then washed with water and eluted with a saturated solution of KCl. The concentration of the purple product of Knoop's reaction on the eluate is measured photometrically.

Results obtained by this method were: Normal individuals differed greatly in the daily amounts of urinary histidine excreted—values from 0–400 mg. per day were obtained. The excretion of histidine fluctuated throughout the day and seemed related to the urinary output. Early morning specimens of urines from normals contained 0–30 mg./100 ml. In pregnancy, early morning specimens contained usually 30–100 mg./100 ml. In pregnancy complicated by toxaemia, threatened abortion or diabetes, the urinary histidine fell to values below the normal. From the present data, it would seem that estimation of urinary histidine is a useful rather than a reliable diagnostic aid in pregnancy.

* Kapeller-Adler, R. & Adler, E. [1943]. *J. Obstet. Gynaec.* **50**, 177.
† Armstrong, A. R. & Walker, E. [1932]. *Biochem. J.* **26**, 143.

The Osmotic Pressure of Foetal Haemoglobin. By E. F. McCarthy and G. Popják

The oxygen dissociation curves of foetal and maternal haemoglobin solutions with identical concentrations of hydrogen ions and salts, have been shown to differ markedly in position. In addition there seems to be a slight difference in the shape of the curves which may be represented by the calculation of n in Hill's equation $y/100 = kx^n/(1+kx^n)$, in which y represents percentage saturation, x oxygen tension and k is a constant. The value of n for foetal haemoglobin is usually slightly lower than that for maternal haemoglobin and it seemed desirable therefore to compare the degree of aggregation of foetal and maternal haemoglobin by osmotic pressure measurements.

Oxygen dissociation curves were measured on solutions of foetal and maternal haemoglobin prepared from the corresponding bloods of sheep at an early stage of pregnancy. Low values of n were obtained for foetal haemoglobin. Osmotic pressure measurements made by Adair's method showed no significant difference between foetal and maternal haemoglobin.

The Osmotic Pressure of 'Defatted' Serum. By G. Popják and E. F. McCarthy

Fresh, pooled human serum was 'defatted' by the method of McFarlane, i.e. by shaking it with ether, then freezing the mixture to below $-25°$ and thawing. The osmotic pressure of serum extracted five times in this way was compared with that of the untreated serum. The osmotic pressure measurements were made by the method of Adair. The results showed that the defatted serum exerts slightly higher osmotic pressure per gram of protein than the whole serum. However, this difference can be explained by the increase in the albumin-globulin ratio of the serum after the extraction. The conclusions are drawn that (*a*) extraction of serum by McFarlane's method removes a portion of the globulin complex, but does not alter the colloid osmotic pressure properties of the remaining proteins; and (*b*) the lipids extracted from the serum have no effect on the osmotic pressure of serum proteins.

Alloxan Diabetes (Shaw Dunn) in the Rabbit. By L. L. Ware (introduced by F. G. Young)

Vitamin C Nutrition. The Correlation of Plasma Ascorbic Acid Concentration and Urinary Saturation Tests. By F. T. G. Prunty and C. C. N. Vass

Unless the subject has recently been on an appreciably higher intake of ascorbic acid than he is now receiving, the plasma ascorbic acid concentration is a satisfactory index of the nutritional state with respect to vitamin C. Moreover, this determination is more easily carried out than a saturation test. When a 'state of saturation' is attained the plasma ascorbic acid concentration is greater than 0·8 mg./100 ml. For clinical purposes it is

suggested that a concentration of not less than 0·4 mg./100 ml. should be considered as a normal value. In general the results obtained by this determination are in agreement with those of Harris* in recent surveys.

* Harris, L. J. [1943]. *Lancet*, **1**, 515.

Succinyl Sulphathiazole and Coprosterol Formation. By O. ROSENHEIM and T. A. WEBSTER.

The bacteriostatic action of succinyl sulphathiazole on intestinal coliform bacteria was found to be concurrent with a complete inhibition of coprosterol formation. The drug was administered to rats in a diet on which about 80 % of the total sterols are excreted as coprosterol.* The conclusion that coliform bacteria are responsible for coprosterol formation was negatived by the fact that, in spite of the immediate reappearance of *B. coli* on withdrawal of the drug, the faecal sterols were found to contain only traces of coprosterol.

These and other results throw doubt on the assumed role of intestinal bacteria and suggest the possibility that the fauna rather than the flora of the intestine may be concerned in coprosterol formation. Protozoa and especially species of *trichomonas* are common parasites of the intestine of man and animals and cholesterol has been recognized as an essential growth factor for four species of *trichomonas*.†

We found that carbarsone (*p*-carbamino phenylarsonic acid), which frees the intestine from *trichomonas*, also completely inhibits coprosterol excretion. The correlation, however, appeared to be fortuitous, for rats freed from *trichomonas* by carbarsone excreted considerable amounts of coprosterol on withdrawal of the drug, but only small amounts some time after treatment with succinyl sulphathiazole, which had not freed them of the flagellates. This interference with colesterol metabolism, shared by an antibacterial and an amoebicidal drug, seems to be unconnected with their action on either bacteria or protozoa and remains so far unexplained.

* Rosenheim, O. & Webster, T. A. [1941]. *Biochem. J.* **35**, 920.
† Cailleau, R. (1937). *Ann. Inst. Pasteur*, **59**, 137, 293; (1939) *C.R. Soc. Biol., Paris*, **130**, 1089.

A Study of the Accuracy of Haemoglobin Methods. By E. J. KING, MARGARET GILCHRIST and G. E. DELORY

Three colorimetric haemoglobin methods—alkaline haematin, cyanmethaemoglobin and carboxyhaemoglobin—were tested by comparison with Fe analyses and O_2 capacity determinations. A Leitz colorimeter (Duboscq type) with mercury green light and a photoelectric colorimeter with a Chance green filter were used, with Ilford grey screens as standards. For the alkaline haematin method a haemin standard was also used. Good results were obtained on each colorimeter; photoelectric readings entail less labour. The variation of each specimen from the average colorimetric relation to iron or oxygen was calculated, and these results expressed as standard deviations.

Twelve haemoglobin preparations from horse and human bloods were found to give colours in NaOH agreeing with their iron content, when compared with standard haemin solutions.

On 20 normal bloods the standard deviations from Fe analyses and O_2 capacities were: alkaline haematin, 2·33 and 2·26 %; cyanmethaemoglobin, 1·25 and 2·18 %; carboxyhaemoglobin, 1·70 and 2·49 %; Haldane dilution tube, 3·02 %. On 20 specimens of washed laked cells from miscellaneous hospital bloods the standard deviations were: alkaline haematin, 3·30 and 2·56 %; cyanmethaemoglobin, 2·63 and 2·11 %; carboxyhaemoglobin, 4·14 and 3·32 %.

The cyanmethaemoglobin method is the most accurate; the other two give accuracy satisfactory for most purposes. The alkaline haematin method is preferred for routine work.

[*The Editors of the Biochemical Journal accept no responsibility for the Reports of the Proceedings of the Society. Abstracts of papers read are published as received from the author.*]

PROCEEDINGS OF THE BIOCHEMICAL SOCIETY

The **231st Meeting** *of the Society was held in the Department of Organic Chemistry, The Imperial College of Science and Technology, South Kensington, London, S.W.* 7, *on Saturday,* 13 *November* 1943, *at* 11 *a.m.*

The Meeting was devoted to a discussion on 'The Tetrapyrrolic Pigments', and the following communications were made:

Properties and Function of Natural Tetrapyrrolic Compounds. By D. KEILIN

Tetrapyrrolic compounds are very widely distributed in nature, forming the active or prosthetic groups of several biologically important substances such as chlorophyll, haemoglobin, cytochrome, catalase and peroxidase. They play a fundamental role in photosynthesis, in transport of molecular oxygen and in catalysis of intracellular oxidation reactions.

The best known substance containing a tetrapyrrolic nucleus or a prophyrin is haemoglobin which is an iron-porphyrin-protein compound. By analytic and synthetic methods it has definitely been established that porphyrins are composed of four pyrrol nuclei united by four CH groups and arranged into a flat closed-ring system. In haematin the iron atom occupies the centre of this ring and of the six covalencies of Fe, four lie in one plane and connect it with nitrogens of pyrrol nuclei, while of the two others, one lies above and one below the plane of the molecule, uniting the Fe atom to nitrogenous substances such as proteins. The simplest tetrapyrrolic compound is porphin, and all the natural and artificial porphyrins can be theoretically derived from it by substitution of the H atoms of its pyrrol nuclei by methyl, ethyl, vinyl, formyl, acetic and propionic acids groups.

The porphyrins are strong and highly fluorescent pigments with sharp absorption bands. They have strong light sensibilizing properties, being therefore very toxic to organisms exposed to light. They readily combine with a great variety of metals such as Fe, Cu, Zn, Mg, Co, Mn, forming well-defined metalloporphyrins. Of these compounds only that with Fe, namely, haematin, is capable of combining with nitrogenous substances such as proteins to form either carriers of molecular oxygen (haemoglobins) or intracellular oxidation catalysts (components of cytochrome).

All the biologically important substances containing tetrapyrrolic compounds are conjugated proteins. The protein must not, however, be regarded as a mere colloidal carrier of the prosthetic group but as an integral portion of the chromoprotein molecule determining not only the magnitude but also the nature of the reaction it promotes. Thus the one prosthetic group protohaematin, combined with four different proteins, may give rise to four different substances, e.g. methaemoglobin, catalase, peroxidase, or a component of cytochrome. These highly specific proteins may therefore become one of the limiting factors in the distribution of these biologically active substances. This may explain the haphazard distribution of haemoglobin in invertebrates when one species may contain this pigment while nearly allied forms are devoid of it, or the appearance of a chromoprotein similar to haemoglobin in *Paramecium* and the nodules of leguminous plants.

The fact that tetrapyrrolic compounds have well-defined absorption spectra makes it possible to follow spectroscopically many reactions in which they are involved. In the case of cytochrome, which forms the main link between the intracellular respiratory systems and molecular oxygen, such observations can be carried out upon living cells.

It enables us to follow spectroscopically not only the respiratory activity of living cells but even to localize and determine the mechanism of action of different respiratory inhibitors. It also makes possible the observation of compound formation between a purified enzyme and its substrate.

As to the origin of the tetrapyrrolic compounds, all we can say is that cells of practically every aerobic organism are capable of synthesizing them. Only a few aerobic microorganims living normally in the presence of haemoglobin have lost this property. Their respiration and growth *in vitro* depends, however, on the addition of small amounts of protohaematin or protoporphyrin, which they can use for synthesis of their intracellular haematin catalysts. On the other hand, the anaerobic organisms are completely devoid of tetrapyrrolic compounds and of the property of synthesizing them.

The natural degradation products of haemoglobin are the bile pigments which have the structure of an open tetrapyrrolic chain with one of the porphin CH group missing. It was shown recently that the porphyrin ring of haemoglobin, while still combined with Fe and globin, may undergo an oxidative opening leading to the formation of cholegloblin. This is followed by the removal of an Fe-protein compound (haemosiderin) and the liberation of biliverdin which undergoes reduction to bilirubin. In this way the organism avoids the liberation of highly toxic porphyrins. The easy degradation of porphyrins within the living organism contrasts with their great stability outside the organism when they are protected from light and oxygen. Under such conditions porphyrins and metallo-porphyrins were found preserved for millions of years in crude mineral oils, bituminous oils, coproliths and asphalt.

Varieties of Haemoglobin. By H. Munro Fox

Chlorocruorin, a unique chemical relative of haemoglobin, which is found in the blood of certain marine annelid worms, has a haem and porphyrin differing from those of haemoglobin. The numerous varieties of haemoglobin itself, including myoglobin in muscles, all have the same haem and porphyrin; they differ from one another in their proteins. The blood pigment of vertebrate animals is distinguished from the haemoglobin (erythrocruorin) found in the blood of certain invertebrates by its molecular weight, its isoelectric point and its amino-acid composition; in these particulars chlorocruorin resembles erythrocruorin. The haemoglobins of various kinds of animals differ from one another in crystal form, spectra, oxygen affinity, resistance to alkali, sulphur content, antigenic properties, etc. There are also differences between the haemoglobins of individuals; these are among the few known individual chemical distinctions. More than one haemoglobin has been found to be present in a single individual, either successively during growth, or simultaneously.

The oxygen affinities of the different haemoglobins suit their mode of action. The haemoglobins in foetal blood, in muscle, in the blood of carp and of invertebrates living in oxygen-deficient surroundings have a high oxygen affinity; they take up oxygen even when little is available. The haemoglobins in the blood of active animals, such as trout and birds, have a low oxygen affinity; plenty of oxygen is available from outside and it is given up by the haemoglobin to the tissues at a relatively high pressure. The oxygen affinities of the blood of small invertebrates is at present being studied with an apparatus requiring only a drop of whole blood.

The actual function of haemoglobin in lower animals is not usually obvious, for invertebrates can be gassed with carbon monoxide without succumbing. The best test of its function is to eliminate haemoglobin by means of carbon monoxide and then to measure the oxygen consumption of the animal. This has been done satisfactorily only

with four invertebrates; in three of them the respiratory blood pigment functions as an oxygen carrier at atmospheric pressure of air and at pressures below this, in one it only functions at low oxygen pressures. Care must be taken in these experiments to use insufficient carbon monoxide to poison the cytochrome oxidase.

Haemoglobin is continuously produced and destroyed both in vertebrate animals and in annelid worms. Why is this so? And what keeps the haemoglobin level constant? An excess of haemoglobin is formed in response to environmental oxygen deficiency in mountaineers, in fishes and in *Daphnia*. What is the internal chemical stimulus for this synthesis?

Tetrapyrrolic Compounds in Plants. By R. Hill

With the photosynthetic activity of plants are associated the five fluorescent compounds: chlorophyll *a* and *b*, Mg-phaeoporphyrin, phycoerythrin and phycocyan. In the algae all five pigments occur; chlorophyll *a* is always present but accompanied by another fluorescent pigment often peculiar to a classified group. Phycoerthyrin and phycocyan occur together and were shown by R. Lemberg to belong to the bile pigment series. In the higher plants chlorophyll *a* is always found with chlorophyll *b*; the same association occurs in many algae. The protochlorophyll which sometimes appears in higher plants was shown to be a Mg-phaeoporphyrin by H. Fischer. Direct observations on the presence or absence of chlorophyll *b* in living cells of different algae have been made by D. W. P. Greenham, R. H. Richens and R. Hill; a previously known pigment of the Phaeophyceae they found to be a Mg-phaeoporphyrin.

Cytochrome discovered in the colourless tissues of green plants by D. Keilin can be said to have brought the plant more into the biochemist's ken. A possibility of removing and concentrating the cytochrome system as a whole from certain plant tissues was realized by K. Bhagvat and R. Hill. Catalytic properties of the preparation were examined and found to resemble similar preparations from mammalian muscle. Also oxidation and reduction of the cytochrome could readily be observed in living colourless tissues from a variety of plants. This showed that respiration passes significantly through cytochrome as in the other aerobic organisms originally examined by Keilin.

Finally, if the green plant cell is originally derived from an aerobic cell containing haematin compounds, substances intermediate between haematin and chlorophyll should exist. R. Scarisbrick and R. Hill have examined haem-protein compounds which could be obtained from green plants. In the water soluble extract of leaves from a variety of plants they found cytochrome *c* and another component having α band at 5600 A. This new haemochromogen seems to be characteristic of plant cells but is not confined to the green parts exclusively. It is autoxidizable but not rapidly enough for catalytic properties. It has sharply defined bands and does not combine with CO. In a 50 % alcoholic extract of leaves, however, another haemochromogen was found. This was designated cytochrome *f*. It has different properties from any previously known haemochromogen. The spectrum is very sharp, resembling cytochrome *c* when the latter is cooled in liquid air. Both α and β bands are complex, showing seven maxima in all: α 5550, 5510, 5450 A.; β 5330, 5250, 5140, 5080 A. The substance shows almost complete non-autoxidizability and the redox potential is positive to cytochrome *c*. It seems to be characteristic of green tissue and could be obtained in 10 times the amount of the haemochromogen 5600 A. from a given source, the ratio chlorophyll to cytochrome *f* being about 150:1. This may be regarded as a pigment more characteristic of photosynthetic cells than cytochrome proper and may represent one of the substances which determine the failure of chlorophyll production in the absence of iron.

The Constitution of Catalase. By E. F. HARTREE

In 1930 Zeile and Hellström prepared a fairly pure and high concentrated preparation of catalase which yielded crystalline protohaemin on acid hydrolysis. These authors concluded that catalase consisted of protohaematin combined with a specific protein.

No investigations of the protein have as yet been carried out but in 1935 Stern showed that treatment of catalase with acid acetone yielded a mixture of haematin and biliverdin. Lemberg showed that the biliverdin was derived from an iron-bile pigment group in the catalase. The possibility that the bile pigment was present as an impurity in the catalase preparation appeared to be ruled out when crystalline horse- and ox-liver catalases were split with acetone in the same way to yield a mixture of haematin aud biliverdin. The ratio biliverdin:haematin was found to be approximately constant (1:3) and it was therefore assumed that each molecule of catalase contained one molecule of bile pigment to three of catalase. Refinement of the analytical methods showed, however, that there were significant variations in the 1:3 ratio and that the catalytic activities of purified preparations were proportional to the concentration of haematin iron.

The evidence now available supports the view that the bile pigment groupings in catalase are catalytically inactive and arise through contact of the enzyme with hydrogen peroxide resulting from cellular metabolism. The following points lend further support: (1) crystalline catalase from blood where peroxide formation is much less than in liver is free from bile pigment; (2) the isolation of catalase from horse liver by a method normally yielding a preparation with a 1:3 ratio can sometimes yield crystalline material with an approximately 2:2 ratio; (3) treatment of catalase preparations with hydrogen peroxide can increase the ratio bile pigment:haematin.

The Metabolism of the Blood Tetrapyrrolic Pigments. By J. R. P. O'BRIEN

The metabolic fate of pyrrole pigments is bound up with the synthesis and degradation of haemoglobin. It rests particularly upon the predominating state of the bone marrow and liver. Even in adverse conditions the body can adequately supply the constituents for porphyrin synthesis. Yet globin and iron are strongly dependent upon exogenous supplies. Haemoglobin synthesis is related to the maintenance of the plasma proteins and protein stores. The mode of assemblage of the three components of haemoglobin is still obscure. Much has to be learnt of the structure of haemoglobin although the application of physical methods in the study of haemoglobin and the metallic porphyrins is giving hopeful results. With such information, differences in the properties of corpuscular haemoglobin and purified haemoglobin may be explained and the urgent need for an exact method of determination of this pigment satisfied. Methaemoglobinaemia and 'sulphaemoglobinaemia' due to drugs points to the necessity for knowledge on the conditions maintaining corpuscular haemoglobin in an active state.

In vitro studies on Hb breakdown have outstripped investigations on bile pigments in humans where they are limited to detection and estimation. From the work of Lemberg, Barkan, Fairley and others the stepwise degradation of Hb may involve methaemalbumin, cholebilin, verdohaemochromogens before bilirubin is formed. In the intestine, the changes suffered by bilirubin may yield a complex mixture of chromogens. Both the state of the liver and of the alimentary canal influence the amount of bilirubinoid pigments in the urine and faeces. It is probable that myoglobin contributes to the faecal bilirubinoids as is suggested by the elimination of mesobilifuscin in myopathies. The formation of mesobilifuscin suggests Hb degradation may proceed as far as dipyrryl methenes such as pentdyopent. As we have found, estimation of faecal bilirubinoids does not give a quantitative measure of the rate of haemoglobin breakdown in the body.

There is as yet no real biochemical means of assessing the haematopoietic activity of the bone marrow. In this problem the excretion of the coproporphyrin isomers I and III may ultimately prove useful. As we and others have found, increased excretion of isomer I is associated with increased haemopoiesis, whereas in toxic conditions and aplastic anaemia isomer III predominates. There are difficulties. The daily output of porphyrin fluctuates. Perfect separation of the isomers is difficult so that the relative amounts of the isomers even in normal conditions has not been decided. Melting-point is not a good criterion of purity of individual isomers. Slight variations in health and in the state of the liver may be associated with overproduction of porphyrins. Since the liver plays so great a part in porphyrin and bilirubinoid metabolism, we have made an intensive study of several tests for hepatic function with the ultimate object of obtaining a closer correlation between the state of the liver and pigment metabolism in humans.

The Porphyrinopathies. By C. Rimington

A study of the porphyrinopathies, diseases accompanied by abnormal production or excretion of porphyrin, is essential to a proper understanding of the metabolism of the tetrapyrrolic pigments.

The porphyrins, of which only members of the isomeric series I and III have so far been found in nature, are widely distributed in small quantities throughout the animal and vegetable kingdoms; their metal complexes in the form of haemochromogens connected with cellular respiration are probably ubiquitous. Uroporphyrins are less widely distributed than protoporphyrin or the coproporphyrins. The bones and urine of the fox, squirrel, the shells of some molluscs and the red wing feathers of the African birds, the *Turacos*, all normally contain appreciable quantities of uroporphyrin. Nevertheless, its appearance in man is associated with pathological conditions.

The porphyrinopathies have been principally studied by Garrod, Günther and Fischer, and since 1935 by Waldenström, whose classification of these diseases into (1) porphyrinuria, (2) porphyria congenita, (3) porphyria cutanea tarda, (4) porphyria acuta, is that most generally acceptable. Porphyrinuria, which denotes an increase in porphyrin excreted in the urine, he separates sharply from the remaining groups, since it may be brought about *in any individual* by certain factors, e.g. hepatic dysfunction, poisoning by lead, sulphonamides, salvarsan, etc., whereas all the other porphyrinopathies rest upon an individual and generally familial idiosyncracy.

Porphyria congenita, present from birth, is accompanied by brown discoloration of the bones and light sensitivity. Its occurrence in a herd of bovines has been studied and from breeding experiments the character appears to be transmitted as a mendelian recessive. The majority of cases are males. Determination of the porphyrin excretion shows that, although this is variable, the ratio of the quantity of coproporphyrin to uroporphyrin is approximately constant.

Porphyria acuta becomes manifest about the second or third decade of life and the tendency toward this disease is a familiar character behaving as a mendelian dominant. Relatives of affected persons may, however, excrete appreciable quantities of uroporphyrin in the urine without necessarily suffering from other symptoms of the disease. Onset is usually sudden and the typical course followed is that of severe attacks of abdominal colic with constipation, vomiting, excretion of large amounts of uroporphyrin or a dipyrrimethene precursor of this porphyrin and of a urobilinoid pigment and, finally, development of a progressive paralysis which usually proves fatal.

There are several forms of the disease in which one or other of the main symptoms may predominate. Photosensitivity is usually absent.

Three principal suggestions have been put forward concerning the origin of the porphyrins in disease. Schreus regards them as derived from the degradation of haemoglobin, Duesberg as unutilized products of a deranged synthesis, whilst Fischer considers increased prophyrin production to be an atavistic step, a return to a foetal or primitive type of pigment metabolism.

Our knowledge does not yet permit of a final pronouncement on this matter. The demarcation between normal and pathological is hard to draw and only enthusiastic and painstaking research upon the biochemistry of the tetrapyrrols will supply the solution to the many problems with which we are still confronted.

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The Structure of Arachidonic and Linoleic Acids

By C. L. ARCUS and I. SMEDLEY-MACLEAN, *The Lister Institute, Chelsea Bridge Road, London, S.W. 1*

(*Received 3 September 1942*)

Early in 1940 Shinowara & Brown described the results of the ozonolysis of methyl arachidonate and of its oxidation by $KMnO_4$ in acetone solution. As a result of their experiments they formulated arachidonic acid as $\Delta^{6.10.14.18}$-eicosatetrenoic acid (I):

$$CH_3.CH{:}CH.CH_2.CH_2.CH{:}CH.CH_2.CH_2.CH{:}CH.$$
$$CH_2.CH_2.CH{:}CH.CH_2.CH_2.CH_2.CH_2.CO_2H \quad (I)$$

They emphasized that this structure was not fully confirmed and must be regarded only as a tentative suggestion. Before the appearance of this paper, Dolby, Nunn & Smedley-MacLean had for some time been engaged in elucidating the structure of this acid, using the method of alkaline permanganate oxidation and as their results were entirely at variance with those obtained by Shinowara & Brown, they published a preliminary account [1940] indicating that arachidonic acid was $\Delta^{5.8.11.14}$-eicosatetrenoic acid (II):

$$CH_3.CH_2.CH_2.CH_2.CH_2.CH{:}CH.CH_2.CH{:}CH.$$
$$CH_2.CH{:}CH.CH_2.CH{:}CH.CH_2.CH_2.CH_2.CO_2H \quad (II)$$

The evidence for this structure was that glutaric and succinic acids were the only dibasic acids isolated; the amount of glutaric acid obtained was very small but it gave no depression in melting-point when mixed with the pure acid. From the other end of the molecule a fraction of volatile fatty acids was obtained which behaved like a mixture of valeric and caproic acids. The melting-point of the *p*-bromophenacyl caproate separated by fractional crystallization was not raised to that of the pure compound, and it was considered desirable at some future time to confirm this result. The authors felt justified, however, in proposing the formula (II) for arachidonic acid.

From the biological point of view, the structure of arachidonic acid is of considerable importance. According to the results of Smedley-MacLean & Hume [1941] the rat synthesizes clupanodonic acid (docosapentenoic acid) only if linseed oil acids are supplied, and it is considered probable that these acids are also the precursors of arachidonic acid in the body. If the formula of Dolby *et al.* were correct, the 11 terminal carbon atoms of arachidonic and linoleic acids would be identically linked and the biological synthesis would involve the addition of 2 carbon atoms at the carboxyl end of the molecule and the introduction of double bonds at the 5.6 and 8.9 positions of the C_{20} chain or in the 3.4 and 6.7 positions of the C_{18} chain. On the other hand, the tentative formula suggested by Shinowara & Brown bore no resemblance to that of linoleic acid and did not contain the structure

$$—CH{:}CH.CH_2.CH{:}CH—$$

characteristic of the linseed acids. A comparison of the ozonolysis products of methyl arachidonate and ethyl linoleate was therefore undertaken by the present authors, together with an acetone-permanganate oxidation of the former, and the investigation had been almost completed when a communication from Mowry, Brode & Brown [1942] appeared in which the formula (I) tentatively suggested by Shinowara & Brown was withdrawn and that proposed by Dolby *et al.* (II) entirely confirmed. The later work of the American authors had been carried out on a large scale, using 197 g. of arachidonate in place of 11 g. employed in our experiments, enabling them to isolate relatively large quantities of purified oxidation products.

An eicosatetrenoic acid has been isolated by Toyama & Tsuchiya [1935] from fish oil which gave on oxidation only acids containing 4 carbon atoms. They therefore considered it to be $\Delta^{4.8.12.16}$-eicosatetrenoic acid which contains 4 of the groupings

$$—CH{:}CH.CH_2.CH_2—$$

and in which the characteristic linseed acid structure is absent. This acid has been designated arachidonic acid, but it seems certain that it is not identical with the arachidonic acid prepared from ox suprarenal fat, and it is unfortunate that the same name should be employed. We suggest that the name arachidonic acid be reserved for that originally isolated by Hartley [1909] from pig's liver, to which the constitution $\Delta^{5.8.11.14}$-eicosatetrenoic acid is assigned, and that the name should not be used to describe the acid obtained from fish oil.

A survey of the literature showed that apparently the only work on the ozonolysis of linoleic acid was by Takahashi [1921] whose results cannot be reconciled with the commonly accepted formula. This provided an additional reason for completing the present work. Takahashi ozonized the acid prepared from soya-bean and rice-bean oils; among the products he identified normal butyric acid and the corresponding aldehyde, glutaric acid and its dialdehyde, azelaic acid and its half aldehyde. He therefore represented the double bonds as occurring

in the 9:10 and 14:15 positions (III) instead of in the 9:10 and 12:13 positions (IV) as has been generally accepted:

$$CH_3CH_2.CH_2.CH{:}CH.CH_2.CH_2.CH_2.CH{:}CH.CH_2.CH_2.CH_2.CH_2.CH_2.CH_2.CO_2H \quad (III)$$

$$CH_3.CH_2.CH_2.CH_2.CH_2.CH{:}CH.CH_2.CH{:}CH.CH_2.CH_2.CH_2.CH_2.CH_2.CH_2.CO_2H \quad (IV)$$

Takahashi stated that the tetrabromo- and tetrahydroxy-stearic acids obtained from his linoleic acid were identical with those prepared from the linoleic acid of poppyseed oil. Haworth [1929] prepared linoleic acid from both soya bean and poppyseed oils, and obtained products by oxidation with acetone-permanganate, and with alkaline permanganate, which were in agreement with formula (IV). Birosel [1937] found the linoleic acid prepared from soya bean oil identical with that from cottonseed oil; both gave the normal tetrabromo- and tetrahydroxy-stearic acids. Ethyl linoleate has now been prepared from linoleic acid tetrabromide (M.P. 114–115°), obtained from maize oil, and on ozonolysis it gave products in agreement with formula (IV) ($\Delta^{9.12}$-octadecadienoic acid). We therefore do not confirm the results of Takahashi.

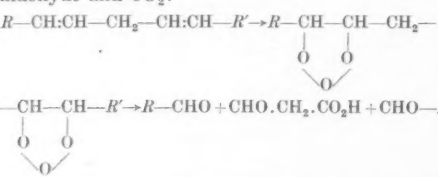
Erdmann, Bedford & Raspe [1909] have ozonized linolenic acid (V) and its esters:

$$CH_3CH_2.CH{:}CH.CH_2.CH{:}CH.CH_2.CH{:}CH.CH_2.CH_2.CH_2.CH_2.CH_2.CH_2.CO_2H \quad (V)$$

They adopted the view of Harries that the ozonide decomposes giving an atom of nascent oxygen for each ozonide group, and that this oxygen may react with H_2O forming H_2O_2 or may oxidize the aldehydes to acids; under these conditions the lower aliphatic aldehydes are those most easily oxidized. Further oxygen is available because linolenic acid and its ethyl ester, when ozonized in chloroform solution, yield the ozonide peroxide; the increase in weight noted indicates this to be the case also for ethyl linoleate. The ozonide of methyl arachidonate was decomposed without removal of solvent but it is likely that it also is an ozonide-peroxide. Erdmann *et al.* [1909] found that the grouping

$$—CH{:}CH.CH_2.CH{:}CH—$$

yielded an ozonide which, with water, gave formylacetic acid, the latter decomposed at 60° to acetaldehyde and CO_2:



In our experiments, on decomposing the ozonides, acetaldehyde was trapped in 2:4-dinitrophenylhydrazine solution and CO_2 in NaOH solution.

Methyl arachidonate was prepared from arachidonic acid octabromide obtained from ox suprarenal fat kindly supplied to us by Messrs Allen and Hanburys Ltd. It had the characteristics recorded in the experimental portion. The observed molecular refractivity was 100·4 units, the calculated value being 99·05. It is considered from this result that in pure methyl arachidonate the double bonds are not conjugated, and that the slight exaltation shown is to be ascribed to the incidence of rearrangement of double bonds into conjugated positions in a fraction of the ester during distillation; this is regarded by Farmer & van den Heuvel [1938] as unavoidable except in molecular stills. The exaltation may be due in part to anomalous dispersion, since the refractive index for λ_{5461} was found to be lower than that for λ_{5893}. The products isolated were identified by the melting-point and by the mixed melting-point with an authentic specimen, the dibasic acids directly, the monobasic acids after conversion into *p*-bromophenacyl esters, and the aldehydes after conversion into 2:4-dinitrophenylhydrazones. The amounts of the products were estimated as far as possible, and where the yields are considerable, though none are nearly quantitative, they are recorded in Table 1; in all three experiments the only volatile acids found were caproic and acetic, and on the assumption that only these were present the quantities given in Table 1 were calculated from the weights and equivalent weights of the various sodium salts.

Table 1. *Ozonolysis of methyl arachidonate*

	Methyl arachidonate	CO_2	Acetaldehyde 2·4-dinitrophenylhydrazone	Na caproate	Na acetate
Wt. (g.)	5·74	0·966	1·976	0·686	2·046
Moles ($\times 10^{-3}$)	18·0	22·0	8·8	5·0	25·0
Ratio	1·0	1·2	0·5	0·3	1·4

Glutaric acid was identified and the presence of caproic aldehyde indicated. The figure given for sodium acetate must be regarded as approximate.

Oxidation of methyl arachidonate by acetone-$KMnO_4$

	Methyl arachidonate 5·0 g. less 1·0 g. recovered	CO_2	Acetic acid	Caproic acid
Wt. (g.)	4·0	2·38	0·86	0·82
Moles ($\times 10^{-3}$)	12·6	54·1	14·3	7·1
Ratio	1·0	4·3	1·1	0·6

Oxalic acid was identified and succinic acid and caproic acid were indicated. A hydrocarbon identified as n-$C_{27}H_{56}$, and polymerized material (0·63 g.) were also isolated.

Ozonolysis of ethyl linoleate

	Ethyl linoleate	CO_2	Na caproate	Azelaic acid
Wt. (g.)	3·37	0·259	0·848	0·753
Moles ($\times 10^{-3}$)	10·9	5·9	6·1	4·0
Ratio	1·0	0·5	0·6	0·4

Acetaldehyde 2:4-dinitrophenylhydrazone was identified.

The caproic acid found in the ozonolysis of methyl arachidonate indicates that the molecule contains the fragment

$$CH_3.CH_2.CH_2.CH_2.CH_2.CH:$$

The non-volatile fraction was easily oxidized by permanganate giving a product containing an ester group and a carboxyl group, and saponification of this material gave glutaric acid as the only product detected. This sequence of reactions is consistent with the conversion of (impure) glutaric half-aldehyde methyl ester into methyl hydrogen glutarate and then to glutaric acid indicating the terminal portion of the molecule to be

$$:CH.CH_2.CH_2.CH_2.COOMe$$

The remaining 9 carbon atoms yielded 1·9 moles of acetyl radical (as acid and aldehyde) which certainly indicated 3 rather than 2 moles. The yield of CO_2 is low, but comparison with the ethyl linoleate experiment points to the presence of 3 moles of CO_2. Having regard to the results of Erdmann *et al.* [1909] three

$$:CH.CH_2.CH:$$

groups are present and must be adjacent. From these results it is concluded that arachidonic acid has the formula (II).

Farmer & van den Heuvel [1938] oxidized with acetone-permanganate a methyl docosahexenoate containing several

$$:CH.CH_2.CH:$$

groups and found that the ethylenic carbons were oxidized to carboxyl so that these groups gave (presumably via malonic acid) acetic acid. Haworth [1929] oxidized linoleic acid with this reagent, and found a similar rupture of the ethylenic groups, but here the malonic acid, traces of which were detected, gave oxalic acid. We have oxidized methyl arachidonate under conditions resembling those of Farmer & van den Heuvel [1938]. The reaction did not proceed smoothly; 20 % of unoxidized ester was recovered after 37 hr., a considerable amount of polymerized material was formed, and a small quantity of a hydrocarbon, identified by melting-point and analysis as n-$C_{27}H_{56}$, was isolated. The last must have been formed under the somewhat strenuous and prolonged reaction conditions; it was not encountered during ozonolysis. It is considered that the methyl arachidonate was slowly oxidized at the double bonds, giving caproic acid, malonic acid (which mainly decarboxylated to give acetic acid but which was also oxidized to oxalic acid) and glutaric acid, which was further oxidized to succinic acid. (The reaction mixture becomes strongly alkaline and apparently hydrolyses the ester group.)

Ozonolysis of the ethyl linoleate yielded caproic acid, indicating the grouping

$$CH_3CH_2.CH_2.CH_2.CH_2.CH:$$

The sequence azelaic half-aldehyde ethyl ester, ethyl hydrogen azelate, azelaic acid could be followed fairly precisely giving the terminal part of the molecule as

$$:CH.(CH_2)_7.COOOEt$$

The acetaldehyde and CO_2 indicate the remaining 3 carbon atoms to have been

$$:CH.CH_2.CH:$$

The commonly accepted formula (IV) for linoleic acid is therefore confirmed.

EXPERIMENTAL

Melting-points and boiling-points are corrected.

Ozonolysis of methyl arachidonate. Methyl arachidonate was prepared by reduction of arachidonic acid octabromide (60 g.) with zinc (60 g.) in methyl alcohol (600 ml.) containing HCl and subsequent esterification according to Ault & Brown [1934]. It (6·25 g.) had b.p. 177–178°/0·35 mm., i.v. Hübl = 310 (calculated for $C_{19}H_{31}COOCH_3$, 319) and $n_D^{24°}$ 1·4828, $n_{5461}^{24°}$ 1·4811, $\delta_{4°}^{20°}$ 0·905, whence R_D obs. = 100·4; R_D calc. 99·0 [Eisenlohr, 1911] or 99·05 [Swietoslowski, 1920].

The ester (5·86 g.) was dissolved in chloroform (40 ml.), cooled in ice and ozonized by passing a current of 8 % ozone through the solution; portions of the solution were withdrawn for test with Br in CCl_4, until (after 22 hr.) no unsaturation remained. Water (45 ml.) was added to the ozonide solution, which was cloudy and smelt aldehydic. The whole was slowly heated to 98° in a distilling flask connected by ground joints to a condenser and a receiver, and thence to a wash bottle containing 600 ml. of saturated solution of 2:4-dinitrophenylhydrazine in 2N HCl, and a long wash tube containing N NaOH (175 ml.) in a column 26 cm. high; CO_2-free air was aspirated through the apparatus. The hydrazine solution was then removed and replaced by fresh reagent and steam passed, aspiration being continued. In this way CO_2, acetaldehyde, non-volatile, and volatile fractions were separated.

The ice-cooled NaOH solution was titrated with N H_2SO_4 using phenolphthalein and then methyl orange, and 0·966 g. CO_2 were found. A slight excess of H_2SO_4 was added and 87 % of the solution was distilled off; the distillate contained volatile acid equivalent to only 2 % of the found CO_2.

The 2:4-dinitrophenylhydrazine solution yielded crude acetaldehyde 2:4-dinitrophenylhydrazone (1·875 g. m.p. of the two ppts. 142–144° and 149–159·5°) from which fractional crystallization gave the pure compound m.p. 169° mixed m.p. 168·5–169° with authenic acetaldehyde 2:4-dinitrophenylhydrazone (m.p. 169°).

The solution of non-volatile compounds was filtered from a small quantity of oily polymer, an equal volume of saturated brine added, and the

solution repeatedly extracted with ether, and dried with Na_2SO_4, yielding 1·53 g. of material. This was suspended in hot Na_2CO_3 solution and treated with 193 ml. $KMnO_4$ ($N/10$) to oxidize aldehyde to carboxyl, on the assumption that 80% would be glutaric half-aldehyde methyl ester. The solution was acidified (H_2SO_4), extracted repeatedly with ether, and the extract washed, dried (Na_2SO_4) and evaporated. The residue was first titrated with, and then quantitatively saponified with, alcoholic potash. (Found: COOMe, 14; COOH, 42%. Calc. for $HOOC.CH_2.CH_2.CH_2.COOMe$: COOMe, 40; COOH, 31%.) The saponified solution, after dilution with water and evaporation of the alcohol, was acidified (H_2SO_4), saturated with salt, and extracted with ether, first by shaking and then for 8 hr. in a continuous extractor. The former extract gave a brown solid which, after crystallization from chloroform-light petroleum, was dissolved in hot water, filtered and evaporated; it then had m.p. 61–65·5° but could not be identified. The second extract gave a white solid which was similarly treated, then repeatedly recrystallized from benzene, and which yielded a small quantity of glutaric acid, m.p. 92·5–93·5° and mixed m.p. 95·5° with authentic glutaric acid (m.p. 97·5–98°).

To the distillate, consisting of chloroform and aqueous layers, more chloroform was added and the whole titrated with N NaOH, with shaking, in a separating funnel. The chloroform solution was separated, dried (Na_2SO_4) and the solvent removed, the latter was mechanically shaken with an excess of a solution of 2:4-dinitrophenylhydrazine in 2N HCl, when it gave a derivative, which after repeated crystallization, had m.p. 152–152·5° (0·101 g.) and was probably impure acetaldehyde 2:4-dinitrophenylhydrazone. The neutral residue, after removal of chloroform, was distilled, giving a small distillate, b.p. 70–130°; this furnished a 2:4-dinitrophenylhydrazone which after recrystallization had m.p. 100–101·5°. The mixed m.p. with authentic caproic aldehyde 2:4-dinitrophenylhydrazone (m.p. 109°) was 103–104°.

The aqueous solution after evaporation and drying to constant weight in a vacuum desiccator, gave 2·732 g. of sodium salt having equivalent 91·3 (from the amount of N NaOH used). (The same procedure for estimating the equivalent of sodium salts was employed a number of times below.) This was dissolved in water (110 ml.), acidified (H_2SO_4) and distilled; the first 26 ml. and the next 75 ml. of distillate were collected. The last was neutralized and yielded a sodium salt (1·378 g., equiv. 83·7), giving a *p*-bromophenacyl derivative which melted for the most part at 81–85° (*p*-bromophenacyl acetate has m.p. 87–87·5°), but which contained a higher melting impurity. We made a number of attempts but were unable to separate the latter. A portion of the sodium salt was refluxed with dilute H_2SO_4 and the solution distilled; the distillate on neutralization gave a sodium salt having equiv. 83·2 (sodium acetate has equiv. 82), but again we were unable to prepare a pure *p*-bromophenacyl ester.

The first fraction of distillate contained a layer of oily acid which was pipetted off, and the solution again distilled; 6·3 ml. of this distillate were taken, combined with the oily acid and neutralized giving 0·403 g. of a sodium salt, equiv. 127. It was converted into its *p*-bromophenacyl derivative which, after three recrystallizations, had m.p. 70·5–71·5° and mixed m.p. 71–71·5° with authentic *p*-bromophenacyl caproate of m.p. 71·5–72°.

Oxidation of methyl arachidonate by acetone-$KMnO_4$. Methyl arachidonate (5·0 g.) was dissolved in dry acetone (300 ml.) and heated under reflux in an apparatus in which the returning acetone percolated through $KMnO_4$. In the course of 37 hr. 36·6 g. of the last were used without the appearance of a pink coloration; since only 31·4 g. would be necessary to convert each ethylenic link into two carboxyl groups, the oxidation was stopped. The solution was filtered from the manganese mud and the latter washed with acetone. The acetone was evaporated and the residue taken up in light petroleum and washed with NaOH solution and with water (added to bulk aqueous extract below). After drying (Na_2SO_4) and removal of solvent, unchanged ester (1·0 g.) remained.

The manganese mud was dried and leached out with 500 ml. boiling water. The latter after filtration, evaporation and desiccation yielded 11·9 g. of salts (A). The desiccated manganese mud now weighed 19·6 g. The CO_2 content of these two fractions (which contained volatile organic acids) was determined as follows. An aqueous solution of the material was heated to boiling under reflux in a current of CO_2-free air, the top of the condenser being connected to a wash bottle containing $N/10$ $Ba(OH)_2$. Dilute H_2SO_4 was run into the solution and the liberated CO_2 swept through. The baryta solution was filtered and titrated against $N/10$ HCl. The difference from a blank gave the CO_2 trapped, and a correction for imperfections in this method was applied from control experiments with known quantities of carbonate. The extracted, dry, manganese mud and the salts (A) contained 48·9 and 119·4 mg. CO_2/g. respectively.

The salts (A) (3·7 g.) were dissolved in water, acidified (H_2SO_4) and steam distilled.

Successive fractions of distillate (ml.)	10	10	10	150	180
Na salt: wt. (mg.)	123	74	55	321	87
equiv.	103·5	98	97	90	172

A repetition with 7·65 g. of salts (A) yielded three fractions of sodium salts: 0·685 g., equiv. 93; 0·211 g.,

equiv. 86; 0·265 g., equiv. 115. The first of these fractions was dissolved in a minimum of water, acidified (H_2SO_4), extracted repeatedly with ether, and the extract dried (Na_2SO_4) and evaporated. The residue was fractionated by distillation through a series of three bulbs which were successively heated in an air-bath. The fraction (20 mg.) taken at air-bath temperature, 195–225°, was converted via the acid chloride to the anilide which had m.p. 79·5–81·0°, mixed m.p. 80–81·5° with caproic anilide (m.p. 96°), and mixed m.p. 55–56·5° with *n*-valeric anilide (m.p. 61·5–62°). The fraction was thus probably caproic acid. The mid-fraction of sodium salts (0·308 g.) yielded a *p*-bromophenacyl derivative (0·721 g.) of m.p. 70°; five recrystallizations gave *p*-bromophenacyl acetate, m.p. 83–83·5° and mixed m.p. 86° with the authentic compound (m.p. 87–87·5°).

There remained in the steam distillation flask a tar and an aqueous solution; the former (insoluble in benzene and in ether) was dissolved in acetone, dried (Na_2SO_4), and after removal of solvent weighed 0·404 g. The aqueous solution was extracted with ether in a continuous extractor for 22 hr. and with fresh solvent for a further 6 hr. On removal of the ether the residue was dissolved in hot acetone (in which nearly all of it dissolved), filtered, and cooled, when crystals, 49 mg., m.p. 55°, were deposited. After two recrystallizations from benzene-acetone, it had m.p. 57–58°. (Found: C, 84·6; H, 15·0 %. n-$C_{27}H_{56}$ has m.p. 58·5–59·5°, C, 85·2; H, 14·9 %.) Addition of two volumes of light petroleum to the acetone filtrate yielded an oil which crystallized. The product after draining and washing with ether had m.p. 99–100° and was dissolved in water, filtered, evaporated and desiccated over H_2SO_4. The residue (45·5 mg.) had m.p. 160–161·5° (decomp.); a portion was converted into the ammonium salt which yielded pyrrole on ignition with zinc dust, indicating succinic acid. A further portion was titrated with NaOH, giving equiv. 51·3, and the solution was acidified (acetic acid), treated with $CaCl_2$ solution, and the precipitate titrated in acid against $KMnO_4$ at 60°, whence 33·2 mg. (anhydrous) oxalic acid were obtained.

The acetone-light petroleum solution on evaporation yielded a residue (C_6H_6), which after washing had m.p. 99–100°. It was dissolved in water, filtered and evaporated and yielded hydrated oxalic acid (41 mg., equiv. 63·2).

Ozonolysis of ethyl linoleate. Corn (maize) oil acids (177 g.; i.v. Hübl = 114) were brominated (40 ml. bromine) in light petroleum solution. The solid product was recrystallized from hot benzene yielding tetrabromostearic acid m.p. 114–115°. It (44 g.) was reduced with zinc dust according to Brown & Fraenkel [1938]. The linoleic acid was esterified with EtOH-HCl and gave ethyl linoleate (12·8 g.), b.p. 173·5°/0·5 mm.; i.v. Hübl = 161 (calc. 164·8); this (3·5 g.) was ozonized as before, saturation being reached after 9 hr. The chloroform was removed as far as possible in a water pump vacuum at 40°, yielding a colourless oil (4·90 g. Theory, assuming a *bis*-ozonide-peroxide, 4·75 g.).

Cold water (35 ml.) was added to the ozonide and decomposition was allowed to proceed in apparatus similar to that used for methyl arachidonate except that five small wash bottles were employed, the first containing a saturated solution of 2:4-dinitrophenylhydrazine in 2*N* HCl, the second the same reagent in alcoholic H_2SO_4 and the last three *N* NaOH (75 ml. each). The water-bath was raised to boiling point over a period of 1 hr. and a slow current of CO_2-free air continued overnight. The contents of the 2:4-dinitrophenylhydrazine wash bottles were then replaced by fresh reagent in 2*N* HCl solution, and steam passed in place of the air current, aspiration through the wash train being continued. Finally the distillate and the solution of non-volatile material in the flask were both saturated with salt and extracted with ether and the latter dried (Na_2SO_4).

The NaOH solution was cooled and titrated with *N* HCl, using phenolphthalein and methyl orange successively and 0·259 g. CO_2 was found.

Of the 2:4-dinitrophenylhydrazine solutions, the alcoholic one gave no appreciable yield, whilst the first and second aqueous ones gave respectively two products (0·151 and 0·035 g.), m.p. 107–109° and 87°, which on recrystallization had m.p. 145·5–147·5° and 137–143°. These were combined and after four recrystallizations had m.p. 160° and mixed m.p. 168–169° with authentic acetaldehyde 2:4-dinitrophenylhydrazone (m.p. 168·5–169°).

Evaporation of the ethereal solution of non-volatile products gave a semi-solid mass which was washed with ether-light petroleum yielding crude azelaic acid (0·122 g.), m.p. 100·5–102·5°. The residue from evaporation of the washings was suspended in hot Na_2CO_3 solution and titrated with $KMnO_4$ until the reaction slowed down (required 63·9 ml. of $KMnO_4$, 0·06*N*). The solution was acidified (H_2SO_4), extracted with ether, the extract washed, dried (Na_2SO_4), and evaporated, giving a semi-solid (1·286 g.). This was dissolved in alcohol and titrated with alcoholic potash and then saponified with excess of this reagent. (Found: carboxyl equiv. 213 and ester equiv. 216; $HOOC.(CH_2)_7COOEt$ has mol. wt. 205). The solution was acidified (HCl), cooled and filtered, yielding crude azelaic acid (0·641), m.p. 102·5–103°. The azelaic acid was recrystallized once from hot water, when it had m.p. 107·5–108° alone, or mixed with authentic azelaic acid (m.p. 106·5–108°).

The ethereal solution of the distillate was titrated, with shaking, in a separating funnel with *N* NaOH. No pure product was isolated from the neutral ether; a carbonyl group was present since reaction occurred with 2:4-dinitrophenylhydrazine but no identifiable hydrazone was isolated; a semicarbazone could not be formed.

The aqueous solution gave a sodium salt (0·875 g.), equiv. 135. This was dissolved in water (55 ml.), acidified (H_2SO_4) and distilled. The first 20 ml. and the next 29 ml. of distillate gave Na salts, 0·380 g., equiv. 136, and 0·317 g., equiv. 132, respectively. The former was converted into its *p*-bromophenacyl derivative which had m.p. 71·5–72° and mixed m.p. 71–72° with authentic *p*-bromophenacyl caproate (m.p. 71·5–72°).

SUMMARY

1. The results of ozonolysis and of acetone-$KMnO_4$ oxidation of methyl arachidonate are in agreement with the constitution (II) $\Delta^{5.8.11.14}$-eicosatetrenoic acid, for arachidonic acid, which was proposed by Dolby *et al.* [1940].

2. Contrary to the results of Takahashi [1921] ozonolysis of ethyl linoleate yields products in accordance with the generally accepted structure for linoleic acid, $\Delta^{9.12}$-octadecadienoic acid (IV).

We desire to express our gratitude to Prof. D. Keilin for hospitality at the Molteno Institute and to Dr F. G. Mann for permission to use the ozonolysis apparatus in the University Chemical Laboratory, Cambridge.

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Studies on Diffusing Factors

10. NOTE ON THE FORMATION OF VISCOUS MATERIALS BY *CLOSTRIDIUM BUTYLICUM*

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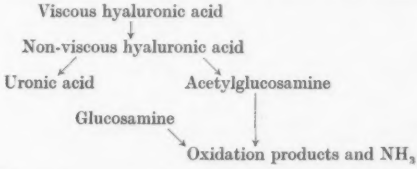
The enzyme system known as hyaluronidase, which exists in some tissues and bacteria, hydrolyses polysaccharides of the hyaluronic acid type. Hyaluronic acid is built up from equimolecular amounts of acetylglucosamine and uronic acid residues [Meyer, 1938]. In the hyaluronidase complex two well-defined individual components have so far been detected [East, Madinaveitia & Todd, 1941]. One of them reduces the viscosity of the viscous polysaccharide without liberating any appreciable amount of reducing sugar. The other is a glucosaminidase which does not attack the viscous polysaccharide but which may play a role in the later stages of the hydrolysis of the polysaccharide by splitting glucosaminic linkages. It is possible that a third component also exists, a uronidase which would hydrolyse the uronidic linkages of the polysaccharide. No evidence has so far been obtained for the presence of such an enzyme in the preparations assayed. The substrate used was menthol glucuronide.

The presence of diffusing factors has been demonstrated in all the sources of hyaluronidase as yet known (e.g. in mammalian testicle, leech extract, snake venoms and some bacteria). It has been claimed that hyaluronidase and diffusing factor are the same substance [Chain & Duthie, 1940; McClean, 1941*b*; McClean & Hale, 1941]. There is, however, a discrepancy between the viscosity-reducing power

and the diffusing potency of various samples assayed [Madinaveitia, Todd, Bacharach & Chance, 1940]. There is no relation at all between the diffusing potency and glucosaminidase activity.

Mammalian testicle possesses, besides hyaluronidase, an enzyme system which deaminates and oxidizes amino sugars [Lutwak-Mann, 1941]. This enzyme system is also present in kidney, brain and in some bacteria. In testicle we find a complete mechanism to utilize the glucosamine present in hyaluronic acid according to the following scheme:



The components of this system which hydrolyse hyaluronic acid have been detected in culture filtrates of bacteria that produce diffusing factor. In this connexion the work of McClean [McClean & Hale, 1941; McClean, 1941*b*] is perhaps significant. He has found that inclusion of hyaluronic acid in the culture media of *Clostridium welchii* and some strains of *Streptococcus* and *Pneumoccocus* enhances the formation of both diffusing factor and hyaluronidase. In the case of *Clostridium welchii* it also increases bacterial growth to about the same extent as glucose. It is of interest to note that no N-acetyl glucosamine could be detected in the medium supplemented by hyaluronic acid in which *Cl. welchii* had grown [McClean & Hale, private communication]. This indicates that the micro-organism has enzymes which, besides hydrolysing hyaluronic acid, also utilize the free glucosamine thus formed. McClean [1941 *a*, *b*] has also observed that addition of hyaluronidase may modify the cultural characteristics of some bacteria. Certain strains of *Streptoccoccus* which in young culture have capsules become non-capsulated when grown in the presence of hyaluronidase. The addition of hyaluronidase to a capsulated strain immediately destroys the capsules. From these capsulated strains a polysaccharide has been isolated [Kendall, Heidelberger & Dawson, 1937] which is similar, or perhaps identical with hyaluronic acid and McClean has found that formation of capsules and production of hyaluronidase do not coexist in the strains examined. In the case of *Pneumococcus* the polysaccharide from which the capsules are formed [Heidelberger, Kendall & Sherp, 1936] although similar, is not identical with hyaluronic acid, and accordingly inclusion of hyaluronidase in the culture medium does not affect capsulation.

The production of polysaccharides by micro-organisms is by no means always connected with capsule formation. A number of bacteria, for example, elaborate viscous substances which they secrete into the culture medium. Whilst conducting biotin assays in connexion with other work in this laboratory, using as test organism *Cl. butylicum* [Snell & Williams, 1939; Peterson, McDaniell & McCoy, 1940] one of us observed that this spore-bearing anaerobe makes the medium in which it is growing viscous. *Cl. butylicum* grows in relatively simple glucose media using asparagin as the only source of nitrogen. Biotin is an essential factor for its growth; this can be supplied either as the pure substance [Snell & Williams, 1939; Woolley quoted by du Vigneaud, Hofmann & Melville, 1941] or in the form of peptone [McDaniell, Woolley & Peterson, 1939].

Since certain of the cultural characteristics of this micro-organism are fairly easily reproducible, it seemed of interest to investigate the factors affecting the production of viscous materials. While we have not been able to obtain evidence for the presence of glucosamine in the viscous material, nor is the viscosity of the latter destroyed by hyaluronidase, yet the growth of *Cl. butylicum* and the formation of viscous material are both inhibited to an appreciable extent by growing the organism in the presence of purified hyaluronidase preparations.

The formation of viscous materials is, however, much more markedly affected by other factors such as the initial concentration in the medium of glucose and of the Bactopeptone which was used in our experiments as a source of biotin. There are optimal concentrations of both glucose and peptone for bacterial growth and for production of viscous materials (Fig. 1). At any given concentration of peptone the optimal concentrations of glucose to obtain maximal growth and maximal formation of viscous materials are not very different. However, if the amount of sugar is constant the concentration of peptone which leads to the formation of most viscous material is much lower than that which produces maximal growth. At the highest concentrations of peptone investigated, practically no viscous material was formed, irrespective of the amount of sugar present, and yet the optimal concentration of peptone for bacterial growth had not been reached. This cannot be attributed to all the glucose being metabolized by the bacteria; at high concentrations of glucose a large proportion of the sugar remained unused.

EXPERIMENTAL

Influence of hyaluronidase on the growth of Cl. butylicum. Aseptic conditions were observed throughout this work.

The strain of *Cl. butylicum* used (kindly supplied by Prof. Elizabeth McCoy, Wisconsin, U.S.A.) had been kept for some time in the form of dry spores.

The preparation of hyaluronidase used was prepared from aqueous extracts of bulls' testicle by Pb acetate precipitation followed by $(NH_4)_2SO_4$ fractionation and dialysis [Madinaveitia, 1941]. The solutions were sterilized by filtration through a Seitz filter.

A series of tubes each having 15 ml. medium containing 1 % bactopeptone, 2 % glucose, 0·2 % asparagine, Speakman's [1923] salt mixture and a trace of reduced iron was prepared. Five of the tubes to be used as controls received 1 ml. of a hyaluronidase solution, 10 mg./ml., before being sterilized in the autoclave. It is known that hyaluronidase is thermolabile. Another five tubes received the hyaluronidase after they had been sterilized. All tubes were inoculated with a loopful of a 24 hr. old culture and incubated in a McIntosh and Fildes jar for 60 hr. at 37°. Then the contents of the tubes were centrifuged until clear. The precipitate was washed with water and the washings rejected. The N content of the washed precipitate was determined and this 'Bacterial N' assumed to be a measure of the number of bacteria present. The supernatant was heated 5 min. at 100° and made clear again by filtration through paper. The viscosity was now determined in an Ostwald viscometer (temp. 25°). The reducing material in the supernatant, as determined by the method of Bertrand, was assumed to be the unused glucose. The figures obtained are given in Table 1.

Table 1

	Bacterial N mg./ml.	Specific viscosity	% sugar consumed
Medium + inactivated hyaluronidase (control)	1·75	0·71	87
	1·68	0·79	92
	1·85	0·93	100
	1·86	0·87	97
	2·01	0·77	97
Medium + hyaluronidase	1·23	0·43	67
	1·29	0·46	79
	—	0·44	79
	1·29	0·51	75
	1·18	0·50	94

Influence of glucose and peptone on the growth of Cl. butylicum. To obtain an indication of the time of incubation required to attain maximal growth, the following experiment was carried out: A series of tubes containing 10 ml. medium (2 % A.R. glucose, 0·6 % Bactopeptone, 0·2 % asparagine, Speakman's salt mixture and reduced iron) was inoculated with a loopful of a 24 hr. old culture of *Cl. butylicum*. All tubes were incubated at 37° in the same McIntosh and Fildes jar. Lots of three tubes were removed from time to time and anaerobiosis re-established before proceeding with the incubation. As soon as the tubes were removed the culture was centrifuged and the 'Bacterial N', specific viscosity and the amount of glucose consumed were estimated as previously indicated. The results obtained are recorded in Table 2; mean values are printed in italics.

Table 2

Time of incubation hr.	Specific viscosity	% glucose consumed	Bacterial N mg./10 ml.
14	0·02, 0·04, 0·04	7·8, 11	0·35, 0·62, 0·60
	0·03	*9·4*	*0·52*
21	0·22, 0·22, 0·45	32·5, 37, 39	0·62, 0·69
	0·30	*36·2*	*0·65*
40	1·15, 1·06, 2·69*	85, 92, 77	0·75, 1·03, 1·11
	1·10	*85*	*0·96*
99	1·50, 1·18, 1·48	96, 99·5, 97	1·15, 1·33, 1·08
	1·39	*97·5*	*1·20*

* Not included in the mean.

From Table 2 one can see that the lag phase is about 10 hr. and that the end of the logarithmic phase is reached in about 2 days.

To determine the influence of glucose and of peptone on the growth of *Cl. butylicum* a series of tubes containing 10 ml. medium of various concentrations of Bactopeptone and of glucose was prepared. They all had 0·2 % asparagine, Speakman's salt mixture and reduced iron. They were sterilized, inoculated, and after 70 hr. incubation at 37°, the bacterial N, viscosity and amount of sugar used were determined. All these operations were carried out as previously described. The concentrations of glucose and of peptone which were examined, and whether or not growth was observed in each of the tubes, are recorded in Table 3.

Table 3

Bacto-peptone mg./ml.	Glucose mg./ml.						
	20	30	50	60	80	150	300
2·5	+	.	+	.	.	−	−
5	.	+	.	+	+	.	.
10	.	+	.	+	+	.	.
12·5	+	.	+	.	.	−	−
20	.	+	.	+	+	.	.
25	+	.	+	.	.	−	−
30	.	+	.	+	+	.	.
40	.	+	.	+	+	.	.
50	+	.	+	.	.	−	−

+ = growth; − = no growth.

Fig. 1 is a graphic representation of the values obtained for bacterial N, specific viscosity and glucose consumed. It is a contour map of the surfaces resulting from plotting these values along an axis perpendicular to the plane of the paper. On this plane the concentrations of glucose and of peptone are represented.

SUMMARY

Cl. butylicum produces viscous materials when grown in a glucose-asparagine-mineral salts medium containing Bactopeptone as a source of biotin. Both the growth and the production of viscous polysaccharides are inhibited to a certain extent by inclusion of hyaluronidase concentrates in the medium.

The effect upon some cultural characteristics of *Cl. butylicum* of the initial concentrations of glucose and peptone in the medium has been studied.

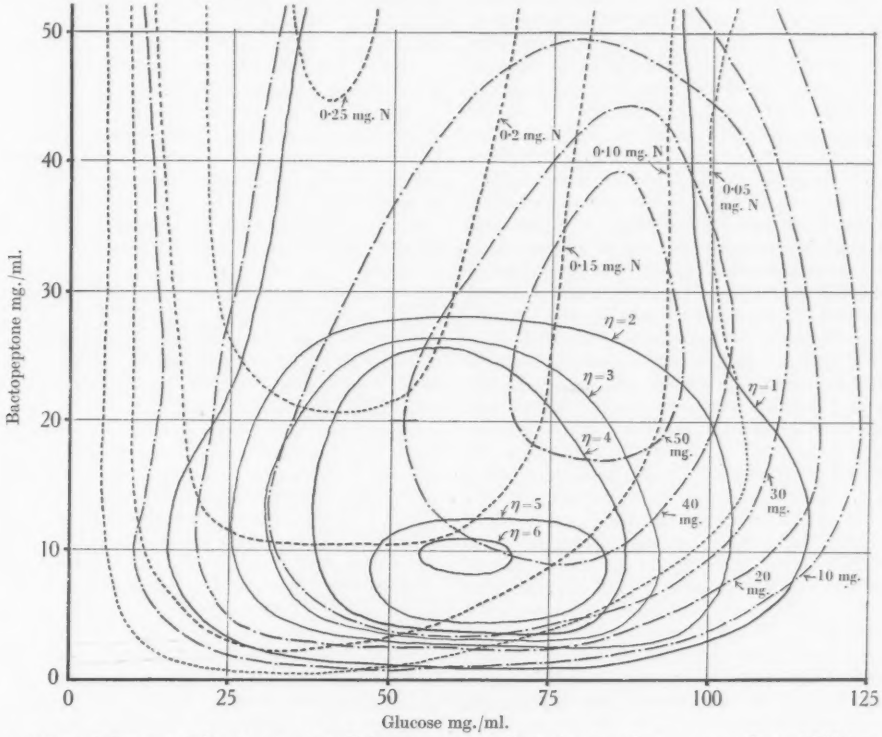


Fig. 1. Effect of glucose and Bactopeptone on *Cl. butylicum*. —— Viscosity of medium. - - - - - Bacterial N (mg./ml.). -·-·-·-·- Glucose consumed (mg./ml.).

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The 'Catalase Test', with Special Reference to *Acetobacter* Species

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The first statement on the occurrence of catalase in bacteria seems to have been made by Beijerinck [1901], who pointed out that whereas the lactobacilli do not contain this enzyme *Acetobacter* species show a positive reaction for catalase. Callow [1923] compared 9 anaerobes and 12 aerobes (none of the aerobes were *Acetobacter* species) with respect to catalase content and noted that the anaerobes were catalase negative and the aerobes were catalase positive. Kluyver [1924], discussing the catalase of micro-organisms, concluded provisionally that this enzyme occurs only in those bacteria which use free oxygen in their metabolic processes. Further information on catalase in bacteria was provided by McLeod & Gordon [1923] and by Virtanen & Karström [1925]. Morgulis, Beber & Rabkin [1926], in their quantitative studies of catalase activity, observed that the activity, which was greatest at 0–10°, diminished with increasing experimental temperatures. The same authors also found that exposure of catalase to temperatures up to 40° for 1 hr. caused practically no inactivation, and cooling the enzyme did not reduce its activity until −10° was reached. The rate of destruction of catalase increased not only with temperature but also with concentration of H_2O_2, and the destruction followed the course of a monomolecular reaction. Experiments with various H_2O_2 concentrations at different temperatures showed that the maximum activity invariably occurred with 0·54*N* H_2O_2. Using catalase from beef kidney they found its activity to be strong over the range *p*H 5–9 with greatest activity between *p*H 6 and 8. The activity was entirely lost at *p*H 2.

Since Beijerinck's early discovery of the presence of catalase in *Acetobacter* species there have been no further reports on the catalase activity of these particular organisms apart from those of Visser't Hooft [1925, 1939], who recorded the presence of this enzyme in *A. aceti*, *A. rancens*, *A. xylinum*, *A. melanogenum*, and *A. suboxydans*, and who described *A. peroxydans*, a species devoid of catalase. No other catalase-negative acetic-acid bacteria have since been reported.

In recent work with 11 species of *Acetobacter* we observed that very erratic results were obtained when cultures were subjected to the 'catalase test'. When well-developed colonies which had been growing at 25° on malt-extract agar (*p*H 5·0) in Petri dishes for 14 days were treated with 0·5*N* H_2O_2, three species gave no response and some of the others reacted only weakly. The results obtained with cultures 8 days' old under conditions which otherwise were identical were practically the same (Table 1).

Table 1

Species of *Acetobacter*	Reaction with H_2O_2 given by cultures aged		*p*H developed by the several species when grown in malt extract (initial *p*H 5·0) at 25° for	
	8 days	14 days	3 days	7 days
(1) *A. aceti*	−	−	3·85	3·60
(2) *A. acetosum*	+	+	3·60	3·25
(3) *A. kuetzingianum*	+	+ +	4·60	4·30
(4) *A. pasteurianum*	+ +	+ +	4·55	4·05
(5) *A. suboxydans*	− ?	−	4·25	3·70
(6) *A. xylinum*	+ + +	+ + +	4·15	3·50
(7) *A. gluconicum*	+	+	4·20	3·75
(8) *A. ascendens*	+ +	+ +	4·60	3·90
(9) *A. capsulatum*	+ ?	−	3·05	2·70
(10) *A. acetigenum*	+ +	+ +	3·80	3·35
(11) *A. turbidans*	+ +	+ +	3·50	3·45

The particular degree of catalase activity shown by each of the several species is probably the resultant of two factors, namely, the quantity of catalase made available by the cells, and the hydrogen-ion concentration produced in the medium by the activities of the cells. Thus, for example, in the cases of the species numbered (4), (6) and (10), which were strong growers and proliferated freely on the slopes, it is probable that the total catalase available was relatively great in amount, hence the influence of the moderate fall in *p*H value was not so obvious and the cultures reacted in a definitely catalase-positive manner. In the case of the species numbered (1) and (5) where growth was relatively scanty, the moderate fall in *p*H had a much greater effect and the cultures proved catalase-negative. Finally, in the case of species number (9), although growth was very abundant it was accompanied by a very considerable fall in *p*H value and the catalase test gave first a doubtful and later a negative result.

The different species were now inoculated into tubes of sterile malt extract (sp.gr. 1·036) buffered with 3 % of $CaCO_3$ which had brought the *p*H to 6·01. After 3 days at 25°, growth was evident in all the tubes, and at this stage in every case the *p*H value

lay between the limits 5·56 and 5·97. Hence such a medium is a satisfactory one for the control of the acidity developed by *Acetobacter* species. Slopes were then prepared from similar buffered malt extract to which 1·5% of agar had been added. Before addition to the extract the $CaCO_3$ had been sterilized in steam and afterwards heated at 160° for 2 hr. to ensure that resistant spore-forming organisms were killed. Cultures grown on these slopes at 25° were all found to give a positive catalase reaction on the 3rd day after inoculation and all still reacted positively on the 14th day.

The following experiment was then undertaken. Malt extract agar, buffered with $CaCO_3$, was poured in separate 10 ml. portions into a set of conical flasks (250 ml.) and sterilized in the usual manner. These flasks were inoculated with the several *Acetobacter* species. In each case a malt-extract culture grown for 3 days at 20° provided the inoculum, 1 ml. quantities being run from sterile pipettes into the several flasks and spread over the surface of the agar by suitable manipulation of each flask. These flask cultures were incubated at 20° for exactly 3 days and estimations of catalase activity were then performed. Into each flask, including an uninoculated 'blank', there were poured exactly 50 ml. of 0·5*N* H_2O_2 (adjusted to *p*H 6·0) and estimations of H_2O_2 were made immediately, then after 15 min., 1 hr., 2 hr., and 3½ hr. These were done volumetrically by the method given by Kolthoff & Sandell [1937]. In the case of the 'blank' the loss (about 10%) of H_2O_2 which was observed up to 45 min. was probably due not to decomposition but to adsorption, but in all the culture flasks much greater losses of H_2O_2 were found to have occurred (Table 2).

Table 2

Acetobacter species	% of added H_2O_2 destroyed after min. 15	60	120	210
A. aceti	19·0	23·5	25·0	25·0
A. acetosum	26·0	29·5	30·0	31·0
A. kuetzingianum	16·0	20·5	21·0	21·5
A. pasteurianum	10·5	35·0	35·5	37·0
A. suboxydans	16·5	30·5	32·5	32·5
A. xylinum	11·5	14·5	16·5	16·5
A. gluconicum	18·5	28·5	30·0	30·0
A. ascendens	12·5	18·0	20·5	21·5
A. capsulatum	23·5	40·0	59·0	75·0
A. acetigenum	10·0	16·5	19·5	19·5
A. turbidans	26·0	36·0	42·0	45·0
	7½	15	30	45
'Blank' Uninoculated medium	3·5	5·5	9·0	10·5

Note. A final set of estimations carried out at 20 hr. from the time of addition of H_2O_2 showed that *A. capsulatum* had destroyed 83·0% and *A. turbidans* 47·0% of the added H_2O_2.

If the percentages of H_2O_2 destroyed are plotted against the time, the relative catalase activities of the organisms which were grown on the buffered medium in the flasks are indicated very clearly and the curves conform well to the shapes of the curves of catalase activity obtained from estimations conducted by Morgulis *et al.* [1926] who used liver catalase. The relatively high degree of catalase activity shown by both *A. capsulatum* and *A. turbidans* is noteworthy; these are two new species both isolated from sour beers and described, respectively, by Shimwell [1936] and by Cosbie, Tošić & Walker [1942]. From this work the following general conclusions can be drawn relating to the catalase activity of *Acetobacter* species: the acid produced by *Acetobacter* species in a medium containing malt extract inactivates catalase. Young cultures which have not passed beyond the early logarithmic phase of growth show a positive catalase reaction even in unbuffered media, because formation of acid does not take place to an appreciable extent until the organisms are well advanced in logarithmic growth. (An exception is *A. peroxydans* which, as mentioned earlier, is catalase-negative.) Older cultures should not be examined for catalase activity unless grown in a buffered medium similar to that described in this paper. The test should be performed as soon as growth has become well established.

Before examining, for catalase activity, new organisms other than *Acetobacter* species, it would seem desirable, in view of our observations, first to determine whether the organisms in question form acid in the medium used and, if they do so, then the cultures prepared for the catalase test should be cultivated in the presence of a suitable buffering agent. It is also necessary to bear in mind that the rate of loss of catalase activity increases with rise in temperature. Hence the cultures prepared for the test should be incubated at the lowest point of the optimum temperature range for that particular micro-organism.

Finally, it may be remarked that the inactivation of catalase by acid as described in this communication is an interesting parallel to the observation of Butlin [1938] that *A. suboxydans* contains as one of its enzyme components an acid-sensitive system which catalyses a reaction in which the O_2 absorbed is equivalent to the CO_2 evolved. In certain conditions this organism cannot exercise this more highly oxidizing function because of the conditions it creates in the cultures if they are unbuffered (e.g. primary production of gluconic acid can occur in a glucose medium of *p*H 6·0 by the agency of another oxidizing enzyme present in *A. suboxydans* and this gluconic acid inhibits the first-mentioned oxidizing system).

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Riboflavin, Vitamin B_6 and Filtrate Factors in Wheaten Flours and Offals

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In recent years there have been many studies of the vitamin B_1 content of wheaten flours and breads, but relatively few estimations have been made of the content of members of the vitamin B_2 complex. Some five years ago Copping & Roscoe [1937] published a survey of the water-soluble B vitamins in yeast, flour and bread, and gave values for the riboflavin and 'factors other than flavin'. These estimations were, however, made at a time when our knowledge of the members of the vitamin B_2 complex was more incomplete than it is at present, and the methods used appear far from satisfactory in the light of later work. New methods for the biological estimation of riboflavin have been described by El Sadr, Macrae & Work [1940], and on the basis of these methods others have now been evolved for estimating vitamin B_6 and filtrate factors. Almost all of the previous values for riboflavin and vitamin B_6 which have been put forward for flours have been obtained by chemical or microbiological methods.

The term filtrate factors is taken to mean all growth factors of the vitamin B_2 complex required by the rat, other than riboflavin, nicotinic acid and vitamin B_6 [El Sadr, Hind, Macrae, Work, Lythgoe & Todd, 1939; Macrae, Todd, Lythgoe, Work, Hind & El Sadr, 1939]. Filtrate factors are now known to include pantothenic acid, biotin and one or more unidentified substances. Lepkovsky [1942] reviews the subject most lucidly.

It is important at the present time to have some precise indication of the nutritive value of the flour and bread available to the people of this country, and on this account, as well as to obtain values by biological methods for comparison with those obtained by other methods, the further study of wheaten flours was undertaken.

MATERIALS

The materials used were supplied through the courtesy of the Research Association of British Flour Millers, who arranged to have suitable flours milled and to store some of the grist so that further samples could be milled if required. In January 1941 the following samples were milled and put into store in a cool place in airtight containers: (1) straight run unbleached white flour of 73% extraction; (2) wholemeal flour of 100% extraction; (3) weatings or middlings; (4) bran; (5) germ. The germ represented all the clean germ obtained in the milling of the white flour, and the bran and weatings together comprised the balance of the wheat after removal of 73% as white flour.

About 6 months later, when the Accessory Food Factors Committee of the Medical Research Council and the Lister Institute had laid down the specification for the preparation of the 85% extraction national wheatmeal [Medical Research Council, 1941], a sample of this type of flour was milled from the original grist and tested in the same way as the other samples.

Table 1. *Composition of the flours and offals* (%)

	Water*	Ash	Nitrogen	Protein
73% flour	13·6	0·411	2·042	11·64†
85% flour	12·64	0·861	2·103	11·98†
100% flour	13·53	1·545	2·190	12·48†
Weatings	14·6	4·31	3·04	17·33†
Bran	13·9	6·55	2·495	15·6‡
Germ	13·98	4·18	4·76	29·75‡

* Dried at 105–110°. † N × 5·7. ‡ N × 6·25.

The vitamin estimations were made on the flours as they were received; they contained about 13% of water (Table 1).

Since the earlier results of Copping & Roscoe [1937] had shown that there was no great difference in the amounts of vitamin B_2 factors present in flours and in the breads baked from them, it was considered unnecessary to bake breads from the flours used for the present investigation, and estimations were made only on the flours.

METHODS

In all tests the criterion was the rate of growth of young rats weaned at 21 days and given the basal diet immediately. In each test comparison was made with a standard preparation of the vitamin in question. Littermate rats, as far as possible of the same sex, were given the doses to be compared, so that for each group of four or more animals on a dose of the cereal product there was a corresponding group on a dose of the standard [Coward, 1938].

The rats received a basal diet containing 60% carbohydrate (maize starch or cane sugar), 20% purified casein, 15% fat and 5% McCollum's normal salt mixture. The fat consisted of 4 parts of hardened arachis oil and 1 part of pure lard. When maize starch was the source of carbohydrate the dry ingredients were mixed with their own weight of water and the mixture was steamed for 3–5 hr., depending on the amount made up, in order to dextrinize the starch and guard against refection [Fridericia, Freudenthal, Gudjonsson, Johansen & Schoubye, 1927]. The sugar-containing diet was not cooked.

The flours tested were made up into similar diets containing 68% flour, 12% casein, 15% fat and 5% salt mixture, and these were cooked in the steamer in the same way as the basal diet. These diets contained approximately the same proportions of carbohydrate, protein and fat as the basal diet and were given as daily doses in weighed amounts, the remainder of the animal's food requirement being made up by giving the basal diet *ad lib.* In the case of bran, difficulty was experienced in making a cohesive mixture with a high proportion of bran in the diet, and a diet containing 30% bran, 30% maize starch, 20% casein, 12% arachis oil, 3% lard and 5% salt mixture was used. The doses of test diets were given in amounts varying from 3 to 10 g. daily, and if any of the test diet was left an equivalent extra amount was given on the next day. As the diets were mixed with their own weight of water the intake of flour was calculated as 34% of the diet eaten. Thus a daily dose of 10 g. of the test diet was equivalent to an intake of 3·4 g. of the flour. Wheat germ was a sufficiently rich source of all the factors tested to be given alone as a separate dose apart from the diet.

Riboflavin estimation. The method was essentially that of El Sadr *et al.* [1940]. The basal diet containing maize starch was supplemented with 0·1 ml. cod liver oil as a source of vitamins A and D, with pure vitamin B_1, and with a whole-liver extract, treated with charcoal to remove the riboflavin, as a source of all the vitamin B_2 factors except riboflavin. Norite charcoal, as used in the original method, was no longer available, but a decolorizing charcoal was substituted and, after some adjustment of quantities, was found satisfactory. In every test two doses of pure riboflavin were given as standard for comparison, usually 6 and 12 μg. per day. The test materials were fed at two levels also, and 4–8 rats were used for each dose level. In the tests with white flour the riboflavin content was so low that the response was too poor to obtain a result of any accuracy; 6 μg. riboflavin were therefore given daily in addition to the flour dose, and the value of the latter deduced by calculation from the curve of response.

Vitamin B_6 (pyridoxine) estimation. The method for the biological estimation of vitamin B_6 was based on that for riboflavin. Rats taken at weaning and weighing about 40 g. were given the vitamin B-free basal starch diet with the addition of daily doses of vitamin B_1 and cod liver oil for 7 days. They were then transferred to a basal diet containing sugar and given additional daily supplements of 40 μg. riboflavin and a fuller's earth filtrate from a liver residue extract. This filtrate supplied the filtrate factor portion of the vitamin B_2 complex; a dose of 1 ml. was equivalent to 6 g. of the original liver. It was found unsatisfactory to give a starch diet in tests for vitamin B_6, as all cereal starches contain some of this vitamin [Chick, Macrae & Worden, 1940]. After some preliminary experiments it was found satisfactory to give the basal diet and supplements other than vitamin B_6 for a standard period of 14 days before administering the test material. The rats usually grew well in the 1st week, but very poorly in the 2nd week, and showed a satisfactory response to a subsequent adequate dose of vitamin B_6. Two levels of dose of pure crystalline vitamin B_6 hydrochloride were given in every test, and the test material was fed at two levels as in the riboflavin tests. This permitted of a good comparison in most cases, since flours and offals were good sources of vitamin B_6.

Estimation of filtrate factors. The filtrate factor portion of the vitamin B_2 complex is known to contain pantothenic acid and at least one other factor [Lythgoe, Macrae, Stanley, Todd & Work, 1940]. An attempt was made to estimate pantothenic acid separately, but so far difficulty has been experienced in devising a basal diet free from pantothenic acid yet containing all other factors of the vitamin B_2 complex. It has, therefore, been possible

only to test for the whole filtrate complex. For this purpose young rats were prepared as for the vitamin B_6 tests and were given the basal sugar diet supplemented with 10 μg. crystalline vitamin B_1, 40 μg. riboflavin, 10 μg. crystalline vitamin B_6 hydrochloride, 1 mg. inositol, 1 mg. nicotinic acid, 3 mg. choline chloride and 0·1 ml. cod-liver oil daily. It was thought advisable to give the additional supplements of inositol, nicotinic acid and choline chloride when the other B_2 vitamins were supplied in the crystalline form. Choline chloride was certainly needed, for it was observed in some tests, before we had seen the reports of György & Goldblatt [1940], that the young rats occasionally died without apparent cause within a week of receiving the basal diet with vitamin B_1, riboflavin and vitamin B_6. Abnormalities of the liver and kidneys, as described by György & Goldblatt, were found in these animals at autopsy, and after choline chloride was given no further losses occurred. As in the vitamin B_6 tests a preliminary period of 14 days with all vitamin B supplements other than filtrate factors was found suitable as a means of preparing the rats for testing. As no source of filtrate factors in a pure form was available a dried baker's yeast was taken as standard of comparison in these tests; it was given at two levels of 100 and 200 mg. daily. The results were calculated in terms of mg.-equivalents of the standard yeast.

Table 2. *Summary of tests for riboflavin content of flours and offals*

Supplement	Daily dose	No. of rats	Av. weekly gain in wt. g.	Calc. content of riboflavin μg./g. fresh wt
1. White flour:				
73% extraction	2·7 g. + 6 μg. riboflavin	14	14	0·85
Riboflavin	6 μg.	14	12	
	12 μg.	14	16	
2. National wheatmeal:				
85% extraction	1·7 g.	8	7·5	2·0
	3·4 g.	8	10	
	2·7 g. + 6 μg. riboflavin	4	16	
Riboflavin	6 μg.	12	11	
	12 μg.	12	15	
3. Wholemeal:				
100% extraction	2·0 g.	4	10	2·3
	4·0 g.	4	14	
	2·7 g. + 6 μg. riboflavin	4	17	
Riboflavin	6 μg.	8	12	
	12 μg.	8	16	
4. Weatings	1·7 g.	4	14	4·0
	2·7 g.	4	15	
Riboflavin	6 μg.	4	13	
	12 μg.	4	16	
5. Bran	1·6 g.	3	14	4·8
	2·16 g.	5	18	
Riboflavin	12 μg.	4	18	
	18 μg.	4	21	
6. Germ I	200 mg.	4	9	7·8
	400 mg.	4	14	
Riboflavin	6 μg.	4	16	
	12 μg.	4	19	
7. Germ II	200 mg.	4	12	12
	400 mg.	4	13	
Riboflavin	6 μg.	3	16	
	12 μg.	3	20	

Table 3. *Summary of tests for vitamin B_6 content of flours and offals*

Supplement	Daily dose	No. of rats	Av. weekly gain in wt. g.	Calc. content of vitamin B_6 hydrochloride μg./g. fresh wt.*
1. White flour:				
73% extraction	1·7 g.	8	11·5	2·5
	3·4 g.	8	15·5	
Vitamin B_6 hydrochloride	2·5 μg.	8	10·5	
	5·0 μg.	8	13	
2. National wheatmeal:				
85% extraction	1·36 g.	8	14	4·2
	2·7 g.	8	18	
Vitamin B_6 hydrochloride	2·5 μg.	8	9	
	5·0 μg.	8	13·5	
3. Wholemeal:				
100% extraction	1·0 g.	8	14	*c.* 5·0
	2·0 g.	8	19	
Vitamin B_6 hydrochloride	2·5 μg.	3	12	
	5·0 μg.	8	13·5	
	10 μg.	4	14·5	
4. Weatings	1·02 g.	4	20	13
	2·04 g.	4	27	
Vitamin B_6 hydrochloride	2·5 μg.	4	9	
	5·0 μg.	4	14	
5. Germ I	200 mg.	4	12	13·6
	400 mg.	4	15	
Vitamin B_6 hydrochloride	2·5 μg.	3	11	
	5·0 μg.	4	15	
6. Germ II	200 mg.	4	9·5	12
	400 mg.	4	15	
Vitamin B_6 hydrochloride	2·5 μg.	4	10·5	
	5·0 μg.	4	14·5	

* These values $\times 0{\cdot}82$ give the actual vitamin B_6 content of the materials.

All results given in the final columns of Tables 2, 3 and 4 are calculated by reference of the weight increase corresponding to the test doses of the unknown substances, to the straight line obtained by plotting the logarithm of the two standard doses to the growth response which they elicited. In no case was use made of a composite curve constructed for the responses to the standard, but in each separate experiment the results were calculated with reference to the line obtained from the results with the standard doses in that particular test.

Table 4. *Summary of tests for filtrate factor content of flours and offals*

Supplement	Daily dose	No. of rats	Av. weekly gain in wt. g.	Calc. content of filtrate factors mg.-equiv. of D.C.L. yeast per g.
1. White flour: 73% extraction	1·7 g.	8	7·5	40–50
	2·7 g.	8	11	
D.C.L. yeast	100 mg.	8	9	
	200 mg.	8	12	
2. National wheatmeal: 85% extraction	1·36 g.	8	10·5	80
	2·7 g.	8	16·5	
D.C.L. yeast	100 mg.	7	10	
	200 mg.	8	15·5	
3. Wholemeal: 100% extraction	1·02 g.	11	11	110
	2·04 g.	11	16·4	
D.C.L. yeast	100 mg.	10	9·7	
	200 mg.	11	15·5	
4. Weatings	1·02 g.	4	15	190
	2·04 g.	4	21	
D.C.L. yeast	100 mg.	4	8	
	200 mg.	4	15	
5. Germ I	250 mg.	4	6	200
	500 mg.	4	10	
D.C.L. yeast	100 mg.	4	11	
	200 mg.	4	17	
6. Germ II	250 mg.	4	5·5	250
	500 mg.	4	9	
D.C.L. yeast	100 mg.	4	8	
	200 mg.	4	14·5	

DISCUSSION

The recorded estimations of riboflavin in cereals include few carried out by biological methods with rats as the test animals. The earlier studies of Copping [1936] and Copping & Roscoe [1937] gave an approximation, but no quantitative values, since factors other than riboflavin were limiting the growth of the rats under the conditions of the tests. Most of the existing values for riboflavin have been obtained by fluorimetric or microbiological methods. Conner & Straub [1941*a*] have reported good agreement in their results obtained by a combined method for estimating vitamin B_1 and riboflavin simultaneously, and by a simple fluorimetric method for estimating riboflavin alone, as used by Hodson & Norris [1939]. Hodson & Norris compared the results obtained with their fluorimetric method for estimating riboflavin with those obtained by the microbiological method of Snell & Strong [1939] and also found good agreement.

All the values reported for wheat and wheat products by the above workers are considerably lower than those found in the present biological estimations. Dr Davis and Mr Newland, of the National Institute for Research in Dairying, Shinfield, have tested the present series of flour samples by a modification of the microbiological method of Snell & Strong and have obtained results in remarkably good agreement with those obtained in the present biological tests (Table 5). Barton-Wright [1942], who used the microbiological method, reports a series of values in good agreement with those obtained by Dr Davis and Mr Newland and by the biological method. Andrews, Boyd & Terry [1942] described studies of riboflavin in cereals by the microbiological method, but their results were much lower than those obtained at Shinfield. It may be that the wheat blend used in milling the present series of flour samples was particularly rich in riboflavin, though this is unlikely. Conner & Straub [1941*a*] report values ranging from 0·89 to 1·91 μg. per g. for 15 varieties of hard wheats and from 0·81 to 1·48 μg. for 16 varieties of soft wheats. The generally higher results obtained by biological methods with rats, as compared with those obtained by fluorimetric or microbiological methods may be due to the difficulty in the extraction of riboflavin from cereal products which is necessary in the latter procedures, or, on the other hand, to the presence of other factors affecting the growth of rats in the biological method. The biological method for estimating riboflavin as originally described by El Sadr *et al.* [1940] has been applied to examining many materials in this laboratory and has been constantly checked by experiments with different doses of pure riboflavin. No evidence has been obtained of the need of the rat for B vitamins other than those present in the liver extract used. The good agreement between the results of biological and microbiological tests on the materials used in this study would also appear to confirm the reliability of the biological method.

Fraenkel & Blewett [1941] demonstrated differences in the riboflavin content of patent, straight run and wholemeal flours by a method in which the criterion adopted was the development of the flour beetle, *Tribolium confusum*. These workers have also demonstrated graduation of riboflavin content in the flour samples used in the present study but have not as yet been able to make a quantitative estimation by their method of riboflavin in such relatively poor sources.

For comparison the riboflavin values obtained for different wheat products by several observers are collected together in Table 5.

Values for the vitamin B_6 content of cereals have not yet been reported to any extent, though it was demonstrated qualitatively some years ago [Birch & György, 1935; Copping, 1936] that most cereals and their milling products were good sources of this nutrient. Swaminathan [1940 *a*, *b*] developed a

Table 5

Material	Riboflavin μg./g. estimated by (1)	(2)	(3)	(4)	(5)	(6)
73% ext. flour	0·85	0·83	0·75–1·25	0·62	0·4	—
85% ext. flour	2·00	2·14	2·00–2·1	—	—	—
100% ext. flour	2·30	2·78	2·65–3·7	1·00	—	0·89–2·03
Weatings (middlings)	4·00	5·90	—	3·80	1·3	—
Bran	4·80	—	5–8	2·80	1·0	3·17
Germ	7·8 and 12	—	9·7–12·85	—	—	3·78–5·56

(1) Copping by biological method in present study.
(2) Davis & Newland by microbiological method on same materials.
(3) Barton-Wright [1942] by microbiological method.
(4) Andrews *et al.* [1942] by microbiological method.
(5) Hodson & Norris [1939] by fluorimetric method.
(6) Conner & Straub [1941*b*] by fluorimetric method.

colorimetric method by means of which he found values of 3·0 and 7·6 μg. per g. for white flour and whole wheat. These values are rather higher than those obtained by the biological method but of the same order. Recently, Conger & Elvehjem [1941] have described a biological method for estimating vitamin B_6 by the growth of rats which is essentially the same as that used in the present study. While these workers did not include cereals among the materials which they examined, so that no comparative values exist, it is worthy of note that the rates of weight increase of their control animals receiving vitamin B_6 hydrochloride in daily doses of 2, 5 and 10 μg. were, respectively, 9, 14 and 19 g. weekly, and in the present tests for rats receiving 2·5 and 5 μg. they were 10 and 14 g. weekly, respectively. This would seem to indicate that the methods in these two laboratories were directly comparable and that biological testing of vitamin B_6 can now be said to be on a sound basis.

The results of the tests for filtrate factors in flours and offals are important in so far as they give a comparison of the value of the different milling fractions and indicate the distribution of the filtrate factors through the grain. This appears to follow that of vitamin B_6 fairly closely, a considerable amount of both vitamins being found in the white flour, with a marked increase in the transition from 73 to 85% extraction and a less marked increase in the further advance to 100% extraction. A great increase in the amount in flour of 85% as compared with that in 73% extraction also held for riboflavin, but the amounts present in the flours and offals were relatively lower than the amounts of vitamin B_6 and filtrate factors. The germ and the weatings or middlings fraction of the grain proved very good sources of all three vitamins, and it is the inclusion of these fractions in the 85% extraction national wheatmeal which raises its nutritive value with regard to all members of the vitamin B_2 complex so greatly above that of white flour.

SUMMARY

1. Estimations have been made by biological methods, with the weight increase of young rats as criterion, of the riboflavin, vitamin B_6, and filtrate factors present in white flour, national wheatmeal and wholemeal, and in wheat germ, bran and middlings or weatings.

2. All the flours were poor sources of riboflavin, but there was a threefold increase with the rise of extraction from 73% for white flour to 85% for national wheatmeal. Wholemeal was only slightly superior to national wheatmeal in its riboflavin content. Wheat germ contained per g. more than three times as much riboflavin as wholemeal, while bran and weatings were about twice as rich in this factor as wholemeal.

3. The differences in vitamin B_6 content in white flour, national wheatmeal and wholemeal were less marked than those in riboflavin content, indicating a more even distribution of vitamin B_6 throughout the grain. Wheat germ and weatings contained per g. about three times as much vitamin B_6 as wholemeal.

4. The amounts of filtrate factors in the flours and offals showed a similar distribution to the amounts of vitamin B_6.

5. The results are summarized in Tables 2–4, and in Table 5 riboflavin values for wheat products obtained by several workers are collected for comparison.

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The Intravenous Glucose Tolerance Equation

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The intravenous glucose-tolerance test in which the blood-sugar is measured at intervals for an hour or two following a single intravenous injection of glucose has received steady but not intensive attention during the last 20 years. Recent important contributions include those of Crawford [1938], Ross [1938], Lozner, Peters, Taylor & Winkler [1941], Hamilton & Stein [1942], McKean, Myers & von der Heide [1935] and Tunbridge & Allibone [1940]. The two last-named papers contain reviews of the literature up to 1935 and 1938 respectively.

The lack of agreement among the various workers regarding procedure is remarkable. This applies not only to the dose of glucose given, but also to the assessment of the results. As the latter involves interpretation of the curve obtained for each test when the blood-sugar is plotted against the time after injection, a study of such curves seems most desirable. This paper concerns curves obtained in a series of glucose tolerance tests performed in this Hospital in 1940 in collaboration with Dr Derek Russell Davis; I undertook recently an examination of them. The following data are important for an appreciation of what follows; fuller practical details are given later in the paper.

In all, 35 tests were carried out on 13 adult psychotic patients. In 26 tests the dose of glucose was 15 g. per sq. m. body surface, in 8 it was 0·5 g. per kg. body weight, and in 1 test it was 27·6 g. [cf. Tunbridge & Allibone, 1940]. The glucose was injected in 33⅓% solution in water over a period of 2 min., and the time at which the blood specimens were taken was measured from the mid-time of the injection. 14 specimens were taken at intervals from 5 to 90 min. (both inclusive) after, and 4 specimens before the administration of glucose. The blood-sugar (in mg./100 ml.) will be denoted by y, the time (in min.) by t, and the graph obtained by plotting y against t will be called 'the curve'. Two of the curves obtained are shown in Figs. 1 and 3 (points represented by circles).

AN EQUATION FOR THE INTRAVENOUS GLUCOSE TOLERANCE CURVE

A simple relationship which might hold approximately is that the rate of fall of blood-sugar at any instant after the injection of glucose is proportional to the value of the blood-sugar at that time, i.e.

$$\frac{dy}{dt} = -Ky, \qquad \text{(i)}$$

where K is a constant. This gives on integration

$$\log y = -Kt + \text{const.} \qquad \text{(ii)}$$

Such a relationship has been found to hold approximately for the disappearance of foreign sugars from the blood [Fishberg, 1930], and also for the disappearance of calcium ion from the blood following

the intravenous injection of calcium salts [Greville, 1931], although in the latter case y represents not the blood-calcium but its excess above the initial value. If equation (ii) holds for the intravenous glucose-tolerance curve, then when log y is plotted against t a straight line should be obtained. In their recent paper Hamilton & Stein [1942] have shown that this is true to a limited degree. The points in the middle part of the plot of log y against t (corresponding to blood-sugars between 100 and 200–250) fall approximately on a straight line; at both ends the points usually deviate, in the direction of increasing y. Now a positive value of y which will be denoted by y_0. The equation which applies is therefore

$$y = -\frac{1}{k}\frac{dy}{dt} + y_0,$$

so that on rearrangement we have instead of equation (i)

$$\frac{dy}{dt} = -k\,(y - y_0), \qquad \text{(iii)}$$

where k, the slope of the line, is given in Fig. 1 by LY/Yy_0. Thus at any instant, the rate

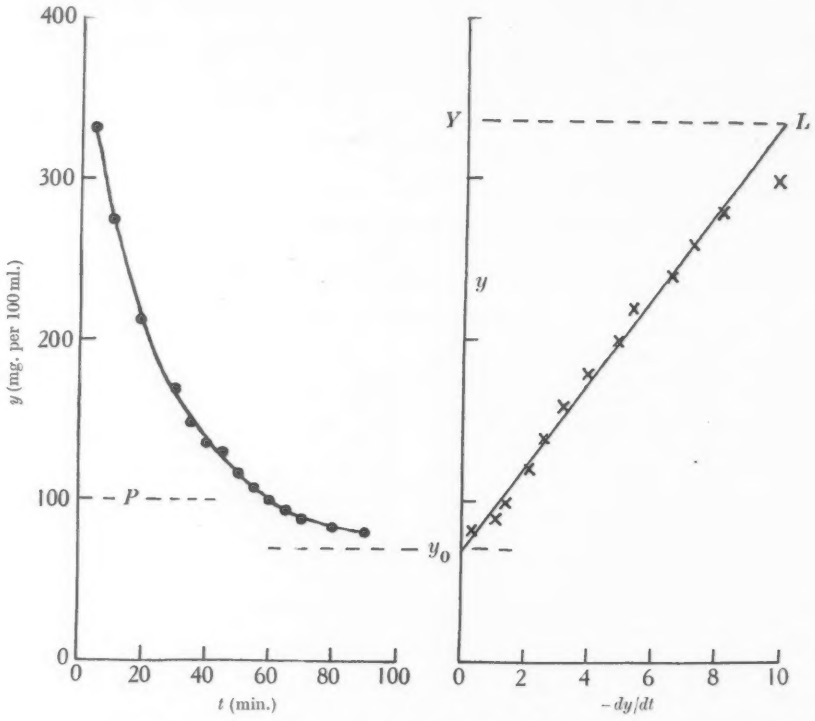

Fig. 1. Intravenous glucose tolerance test. Circles (•), blood-sugar (y) plotted against time (t) (curve fitted by hand). Crosses (×), dy/dt (slope of y, t curve) against y. P = pre-injection blood-sugar. For other symbols see text.

if equation (ii) holds, equation (i) should also apply, in which case dy/dt plotted against y should give a straight line which passes through the origin. In Fig. 1, dy/dt, which is the slope of the y, t curve, has been measured for several values of y, and then plotted against y (crosses). The points fall approximately on a straight line, which, however, does not pass through the origin, but intersects the y-axis at

of fall of blood-sugar is proportional not to the blood-sugar, but rather to the excess of the blood-sugar above some value y_0, which is a constant for the particular test. In that of Fig. 1 y_0 equals 70.

Integrating (iii) we have

$$\log\,(y - y_0) = C - kt, \qquad \text{(iv)}$$

where C is a constant, and expressing equation (iv) in the exponential form,

$$y = e^{C-kt} + y_0. \qquad \text{(v)}$$

Equation (iv) shows that when $\log (y - y_0)$ is plotted against t a straight line should result. This actually occurs, as will be seen from the graph marked '$y_0 = 70$' in Fig. 2. This was obtained by plotting $\log (y - y_0)$ against t, using the data of Fig. 1 and the value of y_0 (70) derived above. The points fall close to a straight line over the whole range of the measured blood-sugar values. A still closer fit is shown in Fig. 3 (graph III) for another test ($y_0 = 52$). With all 35 tests of this series it was found that when $\log (y - y_0)$ is plotted against t the points lie about a straight line for the whole, or nearly the whole, of the range 5–90 min. We may therefore state that, for the series of tests under consideration, equation (iv) represents the data of any test with considerable accuracy; and equation (v) is an intravenous glucose-tolerance equation in the exponential form.

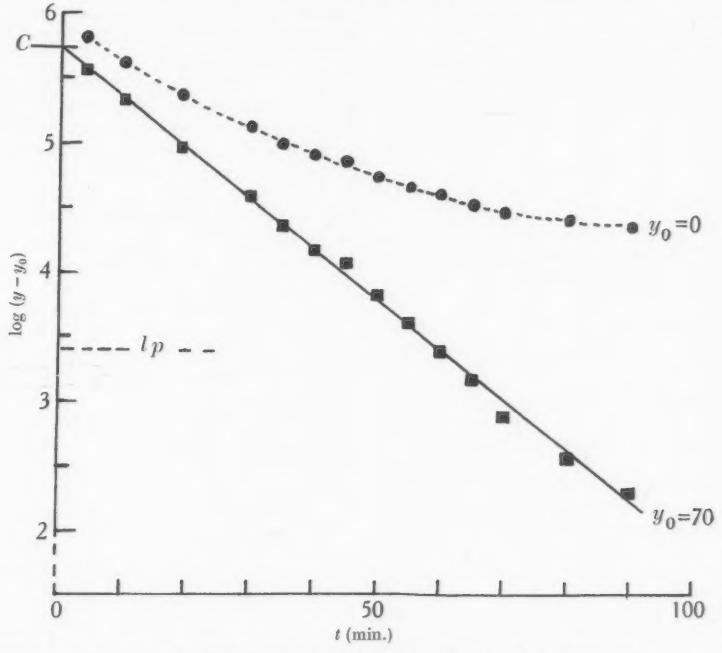

Fig. 2. Same test as Fig. 1. '$y_0 = 70$', $\log (y - 70)$ against t (straight line fitted using constants of regression equation). '$y_0 = 0$', $\log y$ against t. lp: value of $\log (y - 70)$ corresponding to pre-injection blood-sugar.

Estimation of the constants of the equation

Method 1: graphical. This, the method of choice, follows the principles already outlined. y is first plotted against t, and the smooth curve drawn which seems best to fit the points. Then the slope of the curve is measured graphically for several values of y, usually chosen at intervals of 20 mg./100 ml., more frequently where the curve has greatest curvature. It is best to take the mean of two measurements of the slope for each value of y. The values thus obtained for the slope (dy/dt) are plotted against the corresponding values of y, as in Fig. 1. The straight line which best seems to fit the points is drawn; it intersects the y-axis at y_0. y_0 is read off, $\log (y - y_0)$ calculated for each blood specimen, and plotted against t, as in Fig. 2. The points are fitted by eye with a straight line; k is given by the slope of the line, and C by the point at which it intersects the $\log (y - y_0)$ axis.

Method 2: trial and error. $\text{Log}\ (y - y_0)$ is plotted against t several times, using different arbitrary values of y_0 each time, until that value of y_0 is found which brings the points most nearly in a straight line. k and C are then measured from this line as in method 1. Method 2 is somewhat laborious and not very sound, but is occasionally useful.

Method 3: calculation. Let (y_1, t_1), (y_2, t_2), (y_3, t_3) be three points on the curve separated by equal time

intervals, so that $t_2-t_1=t_3-t_2$. Then it can be shown that if equation (iv) holds,

$$y_0=(y_2^2-y_1y_3)/(2y_2-y_1-y_3). \qquad \text{(vi)}$$

I am indebted to Dr Tudor Jones for pointing out this relationship. The method is to read off from the

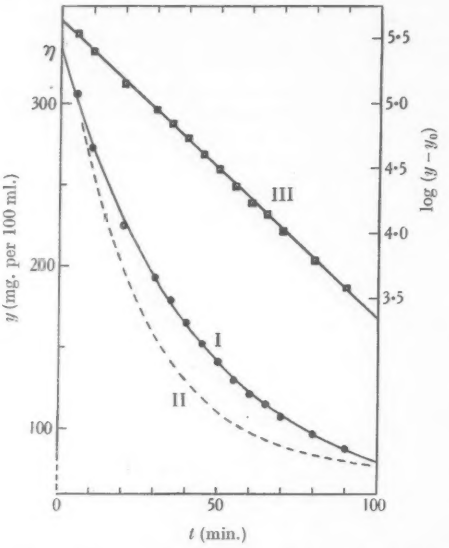

Fig. 3. A different test from that of Fig. 1. Circles (●), observed values of y plotted against t. I (solid curve) represents the equation $y=e^{C-kt}+y_0$, where $C=5{\cdot}638$, $k=0{\cdot}02310$, $y_0=52$. II (broken curve) represents the same equation with k increased to 0·04 and y_0 increased to 72. III, log $(y-52)$ against t (straight line fitted using constants of regression equation).

smooth y, t curve the values of y at (say) 10, 35 and 60 min. y_0 is then calculated by equation (vi). At the same time k can be calculated by the equation

$$k=\frac{1}{t_2-t_1}\log\frac{y_1-y_2}{y_2-y_3}. \qquad \text{(vii)}$$

Equations (vi) and (vii) have, in general, not yielded very accurate results, but once again the method has proved useful on occasion.

Notes on method 1

(*a*) The logarithms in the above equations and figures are of course natural logarithms, to the base e. One can if necessary plot logarithms to the base 10, and multiply the values of C and k found from the graph by $\log_e 10=2{\cdot}30$.

(*b*) Fitting a straight line to the $(dy/dt, y)$ points is sometimes a little difficult. There is often a systematic departure from the straight line with the first one or two points where y is large, which is much more marked in this graph than in the log $(y-y_0)$, t plot. These deviating points are best neglected and the line fitted to the remainder. Again, small deflexions of the y, t curve may produce considerable changes in the slope, and the dy/dt, y plot may be slightly sigmoid as in Fig. 1.

In order to get some idea of the error involved in the drawing of the y, t curve and the measurement of slope, the y, t curve was drawn twice for each of 34 tests. Each curve was used to construct a dy/dt, y curve, so that for each test there were two evaluations of y_0. Statistical examination showed that in 95 tests out of 100 the difference between the two evaluations would not be expected to exceed 15 (standard error = 4·89).* Experience showed that when y_0 was used to construct a log $(y-y_0)$, t curve and hence to estimate k, alteration of y_0 by 15 caused an error of about 13·5 % in the estimate of k. This is not great, considering that the range of k in the series is from 0·016 to 0·039.

Another check on the suitability of the graphical method arises from the circumstance that the dy/dt, y graph should have the same slope k as the log $(y-y_0)$, t graph. For each of the 34 tests, the mean of the two estimates of y_0, derived from two drawings of the y, t curve as above, was used to construct the log $(y-y_0)$, t graph and hence obtain an estimate of k. The slopes of the two dy/dt, y graphs also gave two estimates of k, of which the mean was taken. The arithmetic mean of k from the whole series of log $(y-y_0)$, t graphs was 0·0268, and from the whole series of dy/dt, y graphs 0·0270. In any individual case a series of approximations should strictly be made until the two values of k agree satisfactorily, but the first approximation was generally found to give good enough agreement for all practical purposes, and the closeness of the means of the two sets supports the decision to neglect the small differences.

Goodness of fit: statistical examination

In an attempt to assess the goodness of fit, the linear regression equation relating Z to t was calculated, namely,

$$Z=C-kt,$$

where $Z=\log(y-y_0)$, and C and k are constants, $-k$ being the regression coefficient of Z on t. r^2, the square of the correlation coefficient between Z and t, was then found. r^2 is a measure of the fraction of the variability of Z which is attributable to the linear relationship.

The regression equations have been computed for six tests only, as it would be laborious and of no great worth to calculate them for all; the constants can be

* It should be recognized that the standard error used here is not a measure of the accuracy with which y_0 is determined, but a measure of the consistency of results obtained by the graphical method.

estimated satisfactorily by the graphical method. In order to obtain representative figures the tests were divided into three groups, 'best', 'medium' and 'worst', having regard to the amount of scattering of the points round the y, t curve. They were thus probably assessed according to the amount of some of the tests there seem to be systematic deviations which need consideration.

It is difficult to fix any standard with which to decide whether a point deviates from the line. It is best to examine the graphs with a sense of proportion. I have judged as fairly as I could for each

Table 1. *Intravenous glucose-tolerance test protocols, showing simultaneous values of blood-sugar y (mg./100 ml.) and time t (min.), together with the values of the constants y_0, C and k in the equation log $(y-y_0)=C-kt$ calculated for each test*

No.	1		2		3		4		5		6	
Sex, age	F, 47		F, 46		F, 36		M, 38		M, 54		M, 44	
Diagnosis*	*Mel.*		*C.Ps.*		*Mel.*		*Mel.*		*Par.*		*Mel.*	
Weight (kg.)	44·5		52·6		47·2		51·3		64·9		49·4	
Surface area (m.²)	1·43		1·53		1·45		1·59		1·66		1·61	
Glucose injected (g.)	21·3		26·3		21·6		24·0		25·0		24·0	
y_0	63		73		50		78		61		64	
C	5·68		5·64		5·55		5·24		5·56		5·38	
10^4k	304		385		239		208		259		289	
	t	y	t	y	t	y	t	y	t	y	t	y
Initial value	—	94	—	85	—	85	—	87	—	99	—	88
	5	323	5	309	5	314	5	259	5	299	5	256
	10	264	10	262	10	261	10	231	10	257	10	223
	21	220	20	201	20	205	20	197	20	209	20	185
	26	195	30	167	30	178	30	181	30	182	30	151
	37	162	35	149	35	164	35	170	35	168	35	144
	41	142	40	137	40	148	40	159	40	156	40	136
	46	144	45	123	45	140	45	152	45	154	45	125
	51	130	50	110	50	127	50	145	50	129	50	116
	56	115	55	107	60	112	55	139	55	120	55	104
	60	106	60	98	65	104	60	132	60	115	60	97
	65	103	65	97	70	100	65	128	65	110	65	96
	70	98	71	90	80	87	70	120	70	104	70	92
	80	89	80	84	90	82	80	115	80	93	80	86
	85	84	90	84	—	—	90	108	90	88	90	80

* *C.Ps.* confusional psychosis; *Mel.* melancholia; *Par.* paraphrenia.

experimental error. The two most representative tests of each group were then selected. The results are given in Table 2. The calculations were done for the whole of the range 5–90 min., except that in no. 1 the last specimen was at 85 min. Test no. 7 is that represented in Fig. 3; r^2 is here as high as 0·9989.

Table 2. *Constants of the regression equation $Z=C-kt$, where $Z=\log(y-y_0)$*

Group	No. of test	y_0	C	10^4k	r^2
Best	7	52	5·638	231·0	0·9989
	4	78	5·245	207·6	0·9973
Medium	1	63	5·690	306·9	0·9957
	2	73	5·637	384·9	0·9906
Worst	8	63	5·510	335·8	0·9594
	9	55	5·280	217·8	0·9592

Goodness of fit: range

Above we have assessed the goodness of fit of the straight line given by equation (iv) to the data over the whole experimental range. In Figs. 2 and 3 (III) the points lie close to the line throughout, but in test, the first and last points which lie close to the line.

In 10 tests out of 31 the 5 min. point was judged to deviate, in each case above the line, so that the blood-sugar was falling more rapidly than equation (iii) allows. The 10 min. point deviated in only 2 out of 35 tests. The blood-sugar corresponding to the first point lying near the line was noted for each of 35 tests; the average was 275 mg./100 ml. (range 187–332).

In 13 tests the 90 min. point deviated above or below the line; in 19 it did not. The average of the 35 blood-sugar values corresponding to the last point near the line was 90 (range 75–137). In 16 tests out of 30, equation (iv) continued to be obeyed after the blood-sugar had fallen below the pre-injection value, in one case as far as 24 mg./100 ml. below.

Goodness of fit: exponential curve

A demonstration of the goodness of fit consists in reconstruction of the y, t curve. Thus, the constants of equation (iv) for test no. 7 (Fig. 3) having been accurately determined (Table 2), they are inserted

in equation (v) so that y can be calculated for any t. For the blood specimens we have

t	5	10	20	30	35	40	45
y calc.	302·5	275	229	193	177	163·5	152
y obs.	306	273	225	193	179	165	152
t	50	55	60	65	70	80	90
y calc.	140·5	131	122	114·5	108	96·5	87
y obs.	141	130	121	115	107	96	88

In Fig. 3 the solid curve (I) represents equation (v) with the appropriate values of the constants, i.e.

$$y = e^{5\cdot638-0\cdot02310t} + 52,$$

and the circles represent the experimental values of y.

These results speak for themselves.

The constants

y_0, which might be called the 'limiting value of y', is the level which the curve would approach asymptotically were equation (v) to be obeyed when t becomes large. What happens when y actually approaches y_0 cannot be stated; none of the tests were continued after 90 min., and all the 90 min. values of y were greater than y_0. In Fig. 1 the curve seems to be approaching y_0 with precision. In two curves y reached a minimum (approx. 75 and 68) and then rose again. The values of y_0 in these tests were low (25 and 32 respectively), so that y rose long before y_0 was reached, otherwise hypoglycaemic signs might have appeared. y_0 does not appear to be correlated with the pre-injection blood-sugar, but in all tests it was smaller than the latter. y_0 ranged from 25 to 91.

k, as is seen from equation (iii), gives the rate of fall of blood-sugar per unit excess of blood-sugar above the limiting value y_0. The unit is min.$^{-1}$. Throughout the tests there was a slight tendency for k to increase with y_0. k ranged from 0·0176 to 0·0386.

C. If equation (iv) were to hold for values of t approaching zero, then C would be the value of $\log(y-y_0)$ when $t=0$. If the corresponding value of $(y-y_0)$ be denoted by B, and that of y by η, then $\eta = B + y_0$ and equation (iv) becomes

$$y - y_0 = Be^{-kt}. \qquad \text{(viii)}$$

C ranged from 5·12 to 5·82, B from 167 to 337, and η from 234 to 369.

Non-psychotic subjects

As many psychotics show abnormal oral glucose-tolerance curves, the above findings should be controlled by observations on non-psychotic subjects. I have taken data from the literature, thus covering different experimental conditions, and found C, k and y_0 for a considerable number of tolerance tests. The results with a few of these are shown in Table 3. Except with nos. 2, 8 and 9, it was necessary to measure the published graphs to obtain y and t.* Some of the values of y_0 and k in these tests fall outside the range found with the psychotics. The columns headed 'Range of t' and 'Range of y_0' show the range over which equation (iv) was judged to apply. This was wide, especially with nos. 6 and 9.

EXPERIMENTAL DETAILS

In view of Himsworth's well-known work, the patients were kept on a constant carbohydrate intake of 282–299 g. daily. Absence of infection was checked by determinations of the erythrocyte sedimentation rate. The tolerance tests were performed in the morning, after a fast of about 15 hr. The glucose was injected with the aid of a three-way tap fitted to the syringe nozzle, and 0·1 ml. blood specimens were taken from the ear-lobe [see Harrison, 1937].

Blood-sugar was determined by the cerimetric direct titration method of Miller & Van Slyke [1936], which gives values very close to the 'true' blood-

Table 3. *Constants of the intravenous tolerance equation in non-psychotic subjects*

Reference	Glucose injected	y_0	10^4k	C	Range of t	Range of y	Description
(1)	?	96	1068	5·58	2–30	296–106	Normal curve
(2)	0·5 g./kg.	65	334	5·61	2–75	298–88	? children
(3)	0·5 g./kg.	150	686	5·17	3–30	270–171	Child, 11 yr., rheumatism
(4)	30 g.	85	453	5·48	3½–51½	291–110	Normal man, aged 32 yr.
(5)	15 g.	80	555	5·06	1½–44½	226–94	Same subject as no. 4
(6)	27·6 g.	62	375	5·56	2½–64½	305–85	Normal medical student aged 21 yr.
(7)	27·6 g.	105	263	5·61	10–78	319–142	Fractured femur, man, aged 68 yr.
(8)	25 g.	59	289	5·42	5–120	254–67	Normal male, aged 52 yr.
(9)	20 g.	56	334	5·55	4–112	280–62	Heart disease, female aged 15 yr.

(1) Ross [1938], Fig. 17. (2) Crawford [1938], Table 1, case 1, test *a*. (3) Ibid. Fig. 4. (4) Tunbridge & Allibone [1940], Fig. 4, curve I. (5) Ibid. Fig. 4, curve II. (6) Ibid. Fig. 10, curve II. (7) Ibid. Fig. 11, curve I. (8) Lozner *et al.* [1941], Table 1, no. 5. (9) Jörgensen [1926–7], no. A$_{XIV}$.

* Since many workers present their results in the form of diagrams only, it is difficult to use their work for comparison. Protocols of six tests in the present series are given in Table 1, in case any reader would care to use them for checking the assertions made here.

sugar, and about 7 mg./100 ml. lower than the Hagedorn-Jensen values. Methyl violet 5 *B* was found to be an excellent indicator [cf. Vanossi & Ferramola, 1936].

DISCUSSION

Blood-sugar dynamics. The remarkable thing which has emerged is that the rate of disappearance of glucose from the blood, although presumably the resultant of a number of simultaneous processes, conforms with considerable accuracy to a simple equation.

In the first few minutes the blood-sugar is clearly higher than is to be expected from equation (v). In several tests even the 5 min. value of y is greater than η, the value of y at $t=0$ given by equation (v). On the other hand, Table 3 shows that the $1\frac{1}{2}$ and 2 min. points can on occasion lie approximately on the calculated curve. There is, however, an extremely rapid disappearance of glucose from the blood during a short period after the injection.

Thus, with test no. 7 (Fig. 3), 21·4 g. of glucose were injected into a 44·0 kg. woman. The average blood volume of women, according to Harington, Pochin & Squire [1940], is 72·3 ml./kg., so here it would be 3·18 l. The blood-sugar should therefore rise immediately by 673 mg./100 ml.; hence from the pre-injection level of 89 it should rise to 762. For this test, however, η was calculated to be 333, and the 5 min. value of y was 306. Even if the blood volume were as high as 5 l., y would be expected to rise to 517. Several authors have made a somewhat similar calculation (cf. also Greville [1931] on the injection of calcium salts).

A working hypothesis, unsupported by direct evidence, for the events following the injection of glucose is as follows. The blood-sugar reaches a maximum quickly,* and then falls extremely rapidly, partly due to osmotic entry of water into the blood, but mostly to rapid diffusion of glucose from the blood into the tissue fluids. The glucose is rapidly spread over the 'active volume of the tissue fluids' [Burn & Dale, 1924; Orr-Ewing, 1931]. After a time the blood-glucose will come into dynamic equilibrium with the tissue fluid-glucose. It is now suggested that when this occurs, the rate of fall of the blood-sugar conforms to equation (iii). The disappearance of glucose from the blood at this stage mirrors, as it were, the net metabolic removal of glucose in the tissues. Finally, at a low blood-sugar level, processes tending to raise the blood-sugar achieve predominance, the curve rises, and equation (v) no longer holds.

* Although McKean *et al.* [1935] and Fairley [1936] found that the maximum occurs some minutes after the injection, probably owing to slow mixing of the glucose solution with the blood, the curves of Tunbridge & Allibone [1940] and Crawford [1938] show that the maximum usually occurs before the end of the first minute.

Equation (iii) shows that the rate of fall of blood-sugar bears a straight-line relationship to the blood-sugar level. In experiments in which the rate of glucose utilization was estimated at various maintained blood-sugar levels, it has been found that the former bears an approximate straight-line relationship to the latter, both in the eviscerated, hepatectomized dog [Soskin & Levine, 1937] and in the perfused hind-limb of the cat [Lundsgaard, Nielsen & Ørskov, 1938]. Thus the present findings with a rapidly-falling blood-sugar, and the interpretation of these findings, are in conformity with the observations with steady glycaemic levels.

Properties of the curve. It is seen from equation (v) that when y_0 is altered the curve shifts bodily upwards or downwards. k largely determines the shape of the curve. Thus in Fig. 3, the broken curve (II) was obtained by increasing k to 0·04 from 0·0231, the value applying to the solid curve; increasing k has caused the curve to bend more sharply. (y_0 was also increased, to facilitate comparison by raising the curve.) Similarly, decreasing k would straighten the curve somewhat. When C (or B) is altered, the values of $(y-y_0)$ are all changed in the same ratio (equation viii).

Assessment of tolerance tests. The methods which have been used to characterize the results of intravenous glucose-tolerance tests should now be examined in the light of the findings of this paper.

The time taken for the blood-sugar to return to the pre-injection level [Tunbridge & Allibone, 1940], or to 100 mg./100 ml. [Crawford, 1938], or the value of the blood-sugar at a certain time after the injection [Lozner *et al.* 1941] are criteria which do not seem at first to give any information about the shape of the curve, which depends on k. Nevertheless, any or all of these may possibly be correlated with k; indeed, in the series of tests on psychotics there appears to be a rough relationship between k and the time taken for the blood-sugar to return to 95, one tending to increase as the other decreases.

Ross [1938] has used as a criterion the area under the curve bounded by certain arbitrary limits. Despite the criticisms of this by Tunbridge & Allibone [1940], one should not dismiss it before satisfying oneself that the area is not correlated with any of the characteristics of the curve.

The procedure of Hamilton & Stein [1942] needs more detailed comment. These workers plotted log y against t, and found that equation (ii) is approximately obeyed in the middle part of the graph. In Fig. 2, log y has been plotted against t using the data of Fig. 1. The graph is labelled '$y_0=0$', since equation (iv) becomes identical with equation (ii) when y_0 is put equal to zero. It is clearly curved, in contrast to the plot for the same data of log $(y-y_0)$ against t when $y_0=70$. It is true that in several of the other tests the graph of log y against t is much straighter

in the middle part than that in Fig. 2; but an examination of all the tests leaves no doubt that equation (iv) gives a very much better representation of the data than does equation (ii). The constant K of equation (ii) multiplied by 100 was proposed as a measure of the test. Since it then gives the rate of fall of blood-sugar at any instant as a percentage of the blood-sugar at that instant (equation (i)), it would be very convenient. Unfortunately, as we have seen, equation (ii) does not represent the data well, and it is not easy to determine K by drawing a straight line through points lying on a curve.

When the test is used in the diagnosis of disease, any criterion may be taken which proves adequate in practice. For example, Lozner *et al.* [1941] showed that the blood-sugar 2 hr. after the injection of 25 g. of glucose is a reliable guide for distinguishing between benign glycosuria and diabetes mellitus. They established a mean normal value for the 2 hr. blood-sugar, and also found the standard deviation. Any value differing from the mean by more than three times the standard deviation was considered abnormal. Of course in any test the blood-sugar may have passed through a minimum during the first 2 hr., but this does not matter: the criterion is justified by experience.

We have seen that the curve is characterized by three constants, k, y_0 and C. It is unfortunate from the practical point of view that one is not enough. However, the intravenous glucose tolerance test is regarded as a 'delicate instrument' [Ross, 1938], which 'deserves a somewhat more accurate method of measuring' than is obtained by noting the time at which the blood-sugar returns to normal levels [Hamilton & Stein, 1942]. These views suggest that it may prove necessary in the future to characterize experimental curves by the constants of the tolerance equation, though experience may establish a connexion between any one or any combination of the constants and any of the factors, normal and pathological, on which the outcome of the test depends.

SUMMARY

1. In the intravenous glucose-tolerance test, following a single injection of glucose, the value of the blood-sugar (y) is related to the time (t) after the injection by the equation

$$\log (y - y_0) = C - kt,$$

where y_0, C and k are constants, the values of which vary from test to test.

2. This relationship is obeyed with considerable accuracy over all, or nearly all, of the time period 5–90 min. after the injection. It was found to apply in a series of tests on psychotics, and also in numerous tests on normal subjects taken from the literature.

3. The intravenous glucose-tolerance curve thus approximates to an exponential curve of the form

$$y = Be^{-kt} + y_0,$$

where $\log B = C$; and at any instant the rate of fall of blood-sugar is proportional to the excess of the blood-sugar above the 'limiting value' y_0.

4. Methods for determining the constants of the equation are described, and an attempt is made to estimate the goodness of fit.

5. The assessment of the results of intravenous glucose-tolerance tests is discussed in the light of these findings.

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The Assay of Riboflavin in Cereals and other Products

1. MICROBIOLOGICAL ASSAY

2. FLUOROMETRIC ASSAY

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1. MICROBIOLOGICAL ASSAY

The fluorometric methods of estimating riboflavin developed by Hodson & Norris [1939], Najjar [1941] and others have so far proved to be unsatisfactory in our hands when applied to cereals, for reasons referred to in Part 2. The microbiological method introduced by Snell & Strong [1939], however, with suitable modifications has been found to be satisfactory and expeditious.

EXPERIMENTAL

Organism. The organism used was *Lactobacillus helveticus* from Snell & Strong's original strain no. 7469 obtained from the American Type Culture Collection, Georgetown University, Washington, D.C.* A number of different strains of *L. helveticus* were tested for a quantitative response to riboflavin, but with the exception of a strain from Dr J. G. Davies of the National Institute for Research in Dairying, Reading, and which originated in U.S.A., none gave a quantitative response to riboflavin. The majority grew equally well in the absence of the vitamin, while others gave an 'all-or-none' reaction.

The technique employed for the growth of the organism and for the assay of riboflavin was similar to that employed by Snell & Strong with certain modifications which are described below.

Stock cultures of the organism were carried on a yeast-water glucose agar. The cultures were kept in a refrigerator at a temperature of approximately 4° and removed at monthly intervals.

Basal medium. The composition of the riboflavin-free basal medium for the determination of the vitamin was the same as that recommended by Snell & Strong, with, however, the further addition of asparagine, xylose, pantothenic and nicotinic acids. The composition of 250 ml. of the final medium, sufficient for 50 assay tubes was as follows:

50 ml. photolysed NaOH-treated peptone solution; 50 ml. cystine solution; 12·5 ml. of a 1 % asparagine solution; 5·0 g. glucose; 0·25 g. xylose; 2·5 ml. solution A;* 2·5 ml. solution B; 5 ml. yeast supplement and nicotinic and pantothenic acids so that every ml. of the medium contains 0·05 μg. of these two substances.

Media and extracts were always made with glass-distilled water; this precaution is essential.

Preparation of inoculum. The procedure described by Snell & Strong for the preparation of fresh batches of inoculum for assay purposes was not found to be satisfactory, as there was frequently heavy growth in the riboflavin-free blanks. On this account a fresh inoculum was always prepared from the stock agar culture for each set of determinations.

The presence of xylose in the medium has a two-fold action. In the first place the results between replicates were found to be more concordant whether direct titration with 0·1 *N* NaOH or a nephelometric method were employed for the actual determination, and in the second place, in the case of nephelometric determinations there was a larger 'spread' between concentration levels.

The fermentations were carried out in ordinary chemical or bacteriological test-tubes (18 × 150 mm.) and the technique described by Snell & Strong was strictly followed.

Two methods have been used to determine quantitatively the response of the organism to added riboflavin. In the first the turbidity produced by the growth of the bacteria is measured. This procedure is difficult with highly coloured extracts and special precautions have to be taken. In the second method the acid produced during growth is determined by direct titration with 0·1 *N* NaOH, using bromothymol blue as indicator.

If a nephelometric method be employed, then it is necessary on statistical grounds to have five repli-

* We are indebted to Dr J. C. Drummond for obtaining this culture from Washington.

* Solution A is composed of 25 g. K_2HPO_4 and 25 g. KH_2PO_4 dissolved in 250 ml. distilled water. Solution B contains 10 g. $MgSO_4.7H_2O$, 0·5 g. NaCl, 0·5 g. $FeSO_4.7H_2O$. 0·5 g. $MnSO_4.4H_2O$ dissolved in 250 ml. distilled water. Solution B tends to deposit a dark coloured precipitate upon long standing, so that the solution should be well shaken before use.

cates for each level of concentration. Snell & Strong consider that two are sufficient. This number, however, is too small and the results cannot be relied upon. Moreover, for the unknown solutions whose riboflavin content is to be determined there must also be at least three 'blanks' for each concentration level. These 'blanks' are inoculated in the same way as the other tubes and placed in a refrigerator so that bacterial growth is prevented. The readings for the blanks are subtracted from the values obtained for the tubes in which growth has taken place.

The estimation of acid formed during fermentation, however, was found to be the most convenient method. It is simpler and less cumbersome than a nephelometric method and fewer tubes are required for an assay. Here again, Snell & Strong recommend only two tubes for each concentration level. It is, however, from the practical standpoint, much more satisfactory to use three, and this number was always employed in the present investigation.

For the actual titration a comparator was used. The procedure adopted was the same as that described by Cole [1928] for the estimation of amino-acids. Instead of the contents of the tube being washed into a conical flask and then titrated to *p*H 6·8 using a colour-comparison flask to obtain the necessary end-point as Snell & Strong recommend, the titration was carried out in the fermentation tube itself. A great saving of time is effected by this procedure, and the end-point is clearer and more easily determined than with a colour-comparison flask.

With this method the titration is carried out as follows:

A buffer solution of *p*H 6·8 is prepared by mixing 50 ml. of a 0·2*M* solution of KH_2PO_4 with the equivalent of 23·65 ml. of 0·2*N* NaOH, and diluting to 200 ml. 20 ml. of this solution is then pipetted into a test-tube (18 × 150 mm.) and 20 drops of bromothymol blue indicator added. 20 ml. of distilled water is pipetted into another tube. Two of the tubes of which the acid content is to be determined are now taken and to one is added 10 drops of indicator and the tubes are then arranged in the comparator as shown in the following diagram:

A	B
C	D

Tube A contains distilled water and tube B buffer solution + indicator. Tubes C and D contain the assay solutions and to C indicator is added. No indicator is added to D.

Tube C is now titrated with 0·1*N* NaOH, and for every ml. or part of a ml. of standard alkali added an extra drop of indicator is also added, while to tube D the same amount of standard alkali is run in from the burette. The titration is completed when the colours seen through C and D exactly match.

Preparation of extracts. It has been shown by Scott, Randall & Hessel [1941] that starch has a marked effect in stimulating the growth of *L. helveticus*, and as cereals contain a large amount of starch, this must first be destroyed in the extract before the estimation is carried out. Scott *et al.* used taka-diastase for this purpose. In the present investigation, however, taka-diastase was found to be unsatisfactory. The various samples of the enzyme that were tested showed a marked stimulatory action over other hydrolysing agents, such as ptyalin, H_2SO_4 and HCl. The method of extraction that was eventually adopted was to hydrolyse suitable weighed samples of material with 0·25*N* HCl for 15 min. at 15 lb. pressure in an autoclave. For cooked wheaten products, such as bread, the time of hydrolysis must be increased to 30 min. Nevertheless, hydrolysis with ptyalin in place of taka-diastase or refluxing for 1 hr. with 0·25*N* H_2SO_4 [Hodson & Norris, 1939] or with 0·25*N* HCl were all found to give the same results as autoclaving for 15 min. Autoclaving of the material was adopted simply because it was found to be more convenient. After hydrolysis the *p*H of the extract was adjusted to 6·8 with NaOH and made up to the requisite volume. For the great majority of cereals it was found that a 5 g. sample of the finely ground material hydrolysed with 50 ml. of 0·25*N* HCl and made up to 500 ml. was a convenient quantity for assay. For substances with a low riboflavin content, such as patent flours, 10 g. samples were used. When a high riboflavin content was suspected, e.g. germ, 1 g. samples were used. A convenient rule to follow is to have the extract of such a strength that it contains a total of approximately 10–12 μg. of riboflavin. For each assay three different levels of concentration must be used. The results should not differ among themselves by more than 10 %, and the mean of the values is taken as the riboflavin content. In the present investigation the levels of concentration taken were, 2, 3 and 4 ml. of the extract.

The results from the different methods of extraction that were employed are given in Table 1.

Table 1

Sample	Riboflavin content μg./g.
National Straight Run flour (75 % extraction)	
1. Hydrolysis with ptyalin	0·75
2. Refluxed with 0·25*N* H_2SO_4 for 1 hr.	0·75
3. Refluxed with 0·25*N* HCl for 1 hr.	0·80
4. Autoclaved at 15 lb. pressure for 15 min. with 0·25*N* HCl	0·80
5. Hydrolysed with taka-diastase	1·15

Fats, like starch, exert a stimulating effect upon the growth of *L. helveticus*, and substances with a high fat content, such as soybean, maize, germ and milk, must first be defatted before an

extract is made. Cereals with a high fat content are best defatted by extraction in a Soxhlet extractor with light petroleum. Milk should be separated on a centrifuge and the serum shaken up in a separating funnel with ether. Light petroleum is not a suitable fat solvent with milk, as the mixture tends to form an emulsion.

The stimulating effects of fats on *L. helveticus* are shown by the results in Table 2.

Table 2

Sample	Riboflavin content μg./g.
Milk	
1. Raw	1·95
2. Separated	1·66
3. Separated and extracted with petrol ether	1·66
4. Separated and extracted with ether	1·45

EXPERIMENTAL RESULTS

The results of a number of determinations on different varieties of wheat, various fractions of the wheat grain, wheaten flours and other cereals are given in Table 3; figures are based on the normal water contents of the products.

Table 3

Sample	Riboflavin content μg./g.
1. Wheat, English	1·9–2·65
2. Wheat, Manitoba	2·7–2·9
3. Wheat, Australian	2·4–2·65
4. Wheat, Russian	3·7
5. Wheat, Testa	3·9–5·8
6. Wheat, Aleurone layer	6·3–7·55
7. Wheat, Germ. Commercial sample 1	11·35
8. Wheat, Germ. Commercial sample 2	12·85
9. Wheat, Germ. Commercial sample 3	9·7
10. Flour, A roll	0·4–0·5
11. Flour, C roll	0·5–0·6
12. Flour, National Straight Run (75% extraction)	0·75–1·25
13. Flour, National Wheatmeal (85% extraction)	1·7–2·1
14. Barley	2·3
15. Rye	2·65
16. Maize	2·3–2·7
17. Soybean	3·0–4·9

The riboflavin content of samples of baker's yeast and brewer's yeast was also determined.

	Riboflavin content μg./g.
Baker's yeast	23·0
Brewer's yeast	66·0

The possible destruction of riboflavin by baking was investigated. A control loaf and a loaf prepared from the same flour to which 2 μg./g. of synthetic riboflavin had been added were assayed. The crumb was air dried in the dark and in both cases had a moisture content of 6·7 % (Table 4).

Table 4

	Riboflavin content μg./g.
1. Control loaf	2·85
2. Loaf + 2 μg./g. added riboflavin	4·80
3. Recovered riboflavin	1·95

It will be seen from the results in Table 4 that there is no significant destruction of riboflavin in the baking process.

A survey of the riboflavin content of different milling fractions of an English wheat which was specially milled by our colleague Mr A. G. Simpson gave the results in Table 5.

Table 5

Sample; English wheat (dry milled, moisture content 14·0 %)

	Milling fraction %	Riboflavin content μg./g.	Contribution of riboflavin to Straight Run flour %	Contribution of riboflavin in whole wheat %
Break and scratch flours	23·4	0·9	18·8	11·1
Semolina reduction flour	7·2	0·5	3·5	2·1
Middlings (dunst reduction flour 1)	43·6	0·6	23·2	13·7
Middlings (dunst reduction flour 2)	5·4	4·4	22·7	13·4
Middlings (dunst reduction offals, retreated flour)	1·9	6·2	10·7	6·3
Bran and scratch offals, retreated flour	4·1	5·8	21·5	12·7
Straight Run flour (Calculated value)	85·6	1·5 (1·32)	100·4	59·3
Bran	6·0	6·9	—	18·0
Pollards	5·0	6·3	—	13·6
Fine offals	3·4	6·3	—	9·4
Whole wheat (deter.)	100·0	2·4	—	100·3
Whole wheat (calc.)	—	2·3	—	—

This wheat was milled in the laboratory by a special technique designed to give the maximum amount of white flour. It differs from that which can be normally manufactured in commercial practice.

2. FLUOROMETRIC ASSAY

Two major difficulties have been encountered in this estimation when carried out by any of the previously published fluorimetric methods: (1) the presence of colloidal matter and/or fine suspended particles in the final riboflavin solution as prepared for visual or photoelectric assessment of its fluorescence; this

material cannot be removed by centrifuging or normal filtration and its effect in absorbing and scattering the blue exciting light can easily be so great as to mask completely any fluorescence; (2) the initial low concentration of riboflavin in wheat and the majority of the products thereof which causes the fluorescence of final solutions derived from such materials to be too small to be measurable with any degree of accuracy.

The first of these difficulties was overcome by recourse to the procedure of Najjar [1941] in which the riboflavin is extracted by a non-aqueous medium; by this means all suspended material is left in the aqueous layer.

The second difficulty is of a more serious nature—adsorption of the riboflavin from a fairly large quantity of a wheat extract, followed by elution by means of a small volume of liquid was considered to be most worthy of trial as also by this means various interfering fluorescent materials present in the extract might be eliminated.

Freshly precipitated lead sulphide (as recommended by Najjar for urines) was found to be unsatisfactory for wheat or flour extracts; as also was English fuller's earth, even of grades specially selected for adsorption purposes. Through the kindness of Dr B. S. Platt opportunity was afforded of trying out an American fuller's earth of the 'white' variety ('Superfiltrol') and this has proved satisfactory.

The method which follows has been elaborated around the 'Spekker' fluorimeter but may be adapted readily to other types of fluorimeters.

EXPERIMENTAL

20 g. of finely ground wheat, flour or dried bread (or quantities of milling offals containing approx. 20–50 μg. of riboflavin) are taken in a 250 ml. flask and are suspended with shaking in 100 ml. of 0·25N H_2SO_4. Two further flasks are made up in the same way but with the addition of 10 and 20 μg. respectively of riboflavin, conveniently added as 1 and 2 ml. respectively of a 10 μg./ml. standard solution. The flasks are plugged with cotton wool and are autoclaved for 15 min. at 15 lb. pressure. (In order to ensure complete hydrolysis of pre-cooked wheaten products such as bread, it is advisable to prolong autoclaving to 30 min.—see Part I.)

After cooling, the digests are adjusted to approx. pH 5 by the dropwise addition of 30 % NaOH, using brom-cresol green as an external indicator. They are adjusted to a convenient volume (125 ml.) and are filtered through a no. 5 Whatman paper using suction. The filtrate should at this stage be crystal clear.

Three 50 ml. conical centrifuge tubes are prepared, each containing 0·5 g. ($\pm$ 5 mg.) of 'Superfiltrol' and 45 ml. aliquots of each digest are added to them. The fuller's earth is then maintained in suspension for 5 min. by brisk mechanical stirring, a fine glass rod bent at the tip being convenient for the purpose. After centrifuging, the supernatant liquor is carefully poured off and discarded and approx. 20 ml. of distilled water is added to each tube. After agitation for 2 min. in order to wash thoroughly the earth the tubes are again centrifuged and the washing water decanted off. The tubes are then allowed to drain in an inverted position for a few minutes, after which all traces of water adhering to the inside of the tube are wiped away as far as possible with a clean glass cloth.

Elution of adsorbed riboflavin is carried out by the addition of 8 ml. of a reagent with the following composition: 9 vol. of water, 5 vol. of pyridine (redistilled) and 2 vol. of glacial acetic acid. Mechanical stirring is again employed for 5 min., after which the tubes are centrifuged at slow speed for 5 min.

The eluate from each tube is decanted as completely as possible into suitable glass-stoppered flasks or cylinders, with a total capacity of not less than 33–34 ml. (25 ml. measuring cylinders are usually of this total capacity and are convenient for the purpose.)

To each vessel is added 3 drops of 4 % $KMnO_4$ solution; it is mixed and allowed to stand for at least 1 min. to oxidize interfering substances. Two drops of 10 vol. H_2O_2 will then be found sufficient to decolorize the solutions.

5 g. of anhydrous Na_2SO_4 and 20 ml. of n-butyl alcohol are added to each vessel, the vessels are immersed in a water bath to raise the temperature of their contents to about 35–40°, and are then shaken vigorously for 2 min. After cooling, the whole of the supernatant layers, together with as little of the aqueous layers as possible, are decanted into centrifuge tubes and are spun at slow speed for 5–10 min. It will then be found that sufficient of the butyl-alcohol pyridine layer, now perfectly clear, may be decanted to fill the fluorimeter cell. Measurement of fluorescence is made using blue activating light (Wratten no. 47 filter) and an orange filter (Chances) in front of the photocell. A convenient standard green fluorescing solution is a 1 μg./ml. solution of sodium fluorescein in weak alkali.

Blank determinations on each sample are carried out after destruction of the riboflavin contained therein by exposure to the unscreened radiation of a mercury-vapour arc or to direct sunlight for 1–2 hr. according to the method of Najjar.

INTERPRETATION OF RESULTS

Within a limited range of concentrations, Beers' law is obeyed by solutions of riboflavin. As the drum of the 'Spekker' fluorimeter is calibrated logarithmically, a linear ratio is obtained between drum reading and concentration over this range.

Under the conditions outlined above it is found that this linearity of ratio holds, and therefore a straight line may be drawn relating the fluorescences of the three flour extracts to their added riboflavin contents. The 'blanks' of these three extracts will be identical within the limits of experimental error, and this 'blank' figure is used on the extrapolation of the graph from which may be read the amount of riboflavin present in the total quantity of wheat, flour, bread, etc., which was taken in the first place.

In Table 6 are given some results which have been obtained by the method. They include a number of samples which have been assayed by the microbiological method and which appear in Tables 3 and 4.

Table 6

Sample	Riboflavin content μg./g.
1. Wheats, English (1 in Table 3)	1·7–2·3
2. Wheat, mixed grist	2·4
3. Germ (8 in Table 3)	14·5
4. Bran (6·8 μg./g. by microbiological method)	7·4
5. National Wheatmeal sample (*a*)	1·5
6. National Wheatmeal sample (*b*)	2·0
7. Control loaf (1 in Table 4)	2·6
8. Control loaf + 2 μg./g. added riboflavin)	4·4

Duplicate estimations by the method on different days have given results differing on all occasions by less than $\pm 10\%$.

DISCUSSION

The fluorometric method as outlined above is essentially a modification of that of Najjar. No assumption is made that adsorption or elution of riboflavin is complete—the method depends upon keeping volumes and quantities of reagents, etc., constant as between fortified and unfortified extracts, and the use of a standard solution of riboflavin eliminates calculations on such points, which would otherwise be necessary. This method was chosen as being simpler and more expeditious than adopting the directly quantitative system of multiple adsorptions and elutions, though the occasional adoption of such a procedure provides a useful check.

It is found that whereas for one product of fairly constant composition (e.g. National Wheatmeal flour) the gradient of the fluorescence curve is reasonably constant (and consequently it may be found possible, with the sacrifice of a little accuracy, to make riboflavin additions to occasional samples only), this gradient varies markedly from one wheat or milling product to another, and needs to be separately determined on each sample.

For this reason, and despite all efforts which have been made to simplify the fluorometric technique with a view to making it as expeditious as possible, it must be admitted that it is in our experience a very much more laborious and time-consuming method than the microbiological.

The values found by both methods in the present investigation for the riboflavin content of different cereals show that there are considerably greater amounts of this vitamin present in these products (about 10 times) than is indicated by the literature [Fixsen & Roscoe, *Nutr. Abstr. Rev.* 1939–40]. Recently, Andrews, Boyd & Terry [1942] have published figures for the riboflavin content of wheat and wheaten products, as well as other cereals, obtained by the Snell & Strong microbiological method. With the exception of patent flour, the values they obtained are all much lower (approx. half) than those now reported. One explanation for these low figures is that their extracts were too concentrated (5–10 g. made up to 100 ml.). In our experience there is always a falling off in estimated riboflavin if the extract be too concentrated. There is also the further possibility that the *p*H of the extracts was not correctly adjusted. If the *p*H of the extracts be lower than 6·6 the riboflavin values will be found to be too low. Kemmerer [1942] has reported the results of 18 collaborators in a comparison between the fluorometric and microbiological method for the assay of riboflavin in alfalfa meal, wheat and flour. Good agreement was obtained between the various collaborators for alfalfa meal by either method, but extremely wide variations were obtained for wheat and flour, whichever method was used. For example, the values for wheat by the fluorometric method varied between 0·53 and 2·6 μg./g. and with the microbiological method the variations were 0·78–2·7 μg./g., while for flour both fluorometric and microbiological methods gave a range of 0·15–1·5 μg./g. Such extremely wide variations in results between different workers are difficult to understand. In our experience with the microbiological method fluctuations of the order reported by Kemmerer were never encountered.

That the values reported here are reliable is shown firstly by the fact that the determined and calculated values obtained from the different milling samples (see Table 5 above) agree within experimental error. Secondly, the values obtained for the same samples of cow's milk obtained by one of us (E. C. B.-W.) working with the microbiological method and by Dr S. K. Kon of the National Institute for Research in Dairying working independently at Reading with the fluorometric method, agree within experimental error (see Table 7).

Table 7

Sample	Riboflavin content (μg./g.)	
Cow's milk	Microbiological method	Fluorometric method
1	1·6	1·5
2	1·4	1·32
3	1·5	1·4

SUMMARY

The microbiological method of Snell & Strong and the fluorometric method of Najjar for the assay of riboflavin have been improved and modified with particular reference to cereals and cereal products. Agreement between the results obtained by the two methods is good, and figures are presented covering a wide range of such products.

The microbiological method is preferred as it is more rapid than the fluorometric, and requires less material and no specialized apparatus.

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The Effect of Salts on the Formation of Protein Complexes during Heat Denaturation

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The separation of floccules from solutions of proteins denatured by heat is the final and most obvious stage in a process of aggregation. The factors controlling flocculation have been studied in detail by many workers, but little attention has been given to their effects on earlier stages of aggregation. If the heating is insufficient, or other conditions are unsuitable to cause flocculation, stable solutions of heat-changed proteins are obtained. When solutions containing more than one protein are heated in such conditions, aggregation may take place between particles of different proteins, when stable solutions of protein complexes are produced. Such complexes may have properties different from those of either the unheated mixture of proteins or the individual proteins heated separately. For example, such complexes are formed when serum albumin and globulin are heated together; these have been detected by electrophoresis [Van der Scheer, Wyckoff & Clarke, 1941], as well as by changes in the ease of sedimentation during centrifugation, and by changes in precipitability with salts [Kleczkowski, 1941*a*].

Antibodies and protein-antigens can combine to form complexes with other proteins simultaneously undergoing denaturation by heat. Van der Scheer *et al.* [1941] and Krejci, de Spain Smith & Dietz [1941] showed that when antisera are heated, antibodies are found in products with changed electrophoretic mobility, and Kleczkowski [1941*b*] and Bawden & Kleczkowski [1941, 1942] have described changes in the serological behaviour of both antibodies and protein-antigens after heating with unspecific serum proteins. These changes are most striking when 'O'-type antigens, giving granular precipitation with antisera, or their antibodies, are heated in the presence of serum albumin. The complexes formed by such antigens are no longer precipitated by antibodies, although they still combine with them to inhibit the precipitation of any unheated antigens added subsequently. Similarly, the complexes formed by antibodies still combine with the antigens but are unable to precipitate them.

In this paper the effects of salts on the formation of such complexes are described and compared with their effects on the separation of floccules.

MATERIAL AND METHODS

The proteins used for studying the changes in precipitability produced by the formation of complexes were the 'soluble euglobulin' and albumin fractions from rabbit serum. The proteins used as antigens were tomato bushy-stunt virus, and the soluble whole globulin and albumin fractions from human serum. These were all prepared by the methods previously described [Kleczkowski, 1941 *a*, *b*; Bawden & Kleczkowski, 1941].

The antisera used were against tomato bushy-stunt virus, against the whole globulin of human serum and against human serum albumin. They

were produced from rabbits by intravenous injections.

Dialysis was carried out in cellophane sacs for 3 days against distilled water changed three times a day. To prevent any bacterial decomposition of serum proteins, a little thymol was put into the sacs.

The protein solutions were heated in thin-walled tubes immersed in a water-bath.

The precipitability of heated protein solutions was measured by finding the concentration of $MgSO_4$ necessary to cause precipitation. When a precipitate was formed, it was allowed to settle by leaving the fluid undisturbed for 3 days. Then the supernatant was decanted, and the precipitate washed three times by resuspending in $MgSO_4$ solutions of an appropriate concentration and centrifuging. The N content was determined by a micro-Kjeldahl method, and the results expressed in terms of protein by multiplying by 6·25.

RESULTS

The effect of NaCl on the formation of complexes between soluble euglobulin and albumin fractions of rabbit serum

Table 1 shows the results of heating the soluble euglobulin and albumin fractions from rabbit serum separately and together, both in the presence and absence of NaCl. All the solutions were heated for 10 min. at 80° at *p*H 7·0. It will be seen that when heated in the presence of salt a product was formed in the protein mixture, whose precipitability with $MgSO_4$ differed widely from either protein fraction heated separately. The amount of the product was larger than the total euglobulin in the mixture, so that albumin as well as globulin must have contributed to its formation.

Table 1. *The effect of NaCl on interaction between globulin and albumin fractions of rabbit serum during heating*

The amount of proteins in 4 ml. of heated solutions (mg.)		The amount of precipitate formed during heating (mg.)	The amount of precipitate formed with the following concentrations of $MgSO_4$ (% saturation) (mg.)				
Euglobulin	Albumin		30	50	70	80	100
Heating with 1% NaCl							
0	15	0	0	0	0	0	0
5	0	4	.	.	.	.	.
5	15	0	0	0	0	8	.
Heating in salt-free solutions							
0	15	0	0	0	0	0	0
5	0	0	3·2	.	.	.	.
5	15	0	0	4	.	.	.
5	0 mixed after	0	3·2	.	.	.	.
0	15 heating						

*p*H of the dialysed solutions was adjusted to 7·0 by adding 0·1 *N* NaOH. The solutions were heated for 10 min. at 80°.

Serological precipitin tests were made by adding 1 ml. of antigen solutions to 1 ml. of antiserum solutions, so that the range of antigen/antibody ratios, indicated in the tables, was covered. The tubes were immediately placed in a water-bath at 50° with the fluid columns half immersed, so that convection currents ensured complete mixing. The appearance of floccules was taken as a positive result. The ability of treated antigens to combine with antibody without causing precipitation was measured by their ability to inhibit precipitation. After 3 hr. incubation at 50°, appropriate amounts of unchanged antigen were added to the tubes in which there were no floccules. The absence of precipitation after this addition of precipitable antigen was taken as evidence of inhibition by the treated antigen. Similarly, the ability of treated antibody to combine with antigen without causing precipitation was tested by adding untreated antiserum to the tubes in which there was no precipitation.

When the salt-free mixture of the proteins was heated, a product was also formed whose precipitability differed from that of either protein heated separately. This effect was not due to mixing the proteins, for when the two were heated separately and then mixed, their precipitabilities were the same as when tested separately. Thus during heating together both in the presence and absence of salt, there is an interaction between the two proteins leading to formation of a product with new properties. The amount of the product, however, is much greater when the heating is carried out in the presence of salt, and its precipitability then also differs more widely from that of the euglobulin heated alone. These results suggest that in the presence of salt euglobulin combines with considerable quantities of albumin, whereas in the absence of salt it combines with only small quantities to give a product whose properties differ only slightly from those of the euglobulin heated alone.

The effect of salts on the formation of serologically non-precipitating complexes between human and rabbit serum albumins during heating

The euglobulin and albumin fractions of rabbit serum differ so widely in their precipitability with $MgSO_4$ and $(NH_4)_2SO_4$, both before and after heating, that complex formation between them can be readily detected by changes in the precipitability with these salts. This method, however, can obviously not be applied to proteins with similar precipitabilities, such as the albumin fractions from two different animal sera. The formation of complexes often influences the serological reactions of protein-antigens, so that serological methods can be applied for their detection in systems where precipitation with salts is unsuitable. In the tests described below the inability of complexes to precipitate with the antisera, and also their ability to inhibit specifically the precipitation of unchanged

Table 2. *The effect of salts on the formation of non-precipitating complexes between human serum albumin and rabbit serum albumin*

Concentration of salt (normality) in heated solutions	Appearance of the solutions after heating	Precipitin test Human albumin mg. 0·5	0·25	0·12	0·06	0·03	0·015	0·0075
Unheated human albumin		−	+	+	+	+	+	+
Heating at *p*H 7·0								
R Salt-free	clear	+	+	+	+	+	+	+
$Al_2(SO_4)_3$								
R 0·00015	clear	+	+	+	+	+	−	−
R 0·0003	clear	+	+	i	i	0	0	0
R 0·0006	sl.op.	i	i	i	i	i	0	0
R 0·0012	st.op.	+	+	+	+	0	0	0
R 0·0025	pptt.	.	.	.	.	.	.	.
H 0·0006	clear	−	+	+	+	+	+	+
$MgSO_4$								
R 0·0006	clear	+	+	+	+	−	−	−
R 0·0012	clear	+	+	i	i	0	0	0
R 0·0025	sl.op.	i	i	i	i	i	i	0
R 0·005	st.op.	i	i	i	i	i	0	0
R 0·01	st.op.	+	+	+	0	0	0	0
R 0·015	pptt.	.	.	.	.	.	.	.
H 0·0025	opal.	+	+	+	+	+	+	−
Heating at *p*H 3·2								
R Salt-free	clear	+	+	+	+	+	+	+
$BaCl_2$								
R 0·05	clear	+	+	+	+	+	+	+
R 0·2	opal.	i	i	i	i	0	0	0
H 0·2	clear	+	+	+	+	+	+	+

Concentration of salt (normality) in heated solutions	Appearance of the solutions after heating	Precipitin test Human albumin mg. 0·5	0·25	0·12	0·06	0·03	0·015	0·0075
$BaCl_2$								
R 0·0006	clear	+	+	+	i	0	0	0
R 0·0012	clear	+	+	i	i	i	0	0
R 0·0025	sl.op.	i	i	i	i	i	i	0
R 0·005	st.op.	−	+	+	+	+	+	+
R 0·01	pptt.	.	.	.	.	.	.	.
H 0·0025	sl.op.	+	+	+	+	+	+	−
Na_2SO_4								
R 0·012	clear	+	+	+	i	0	0	0
R 0·05	sl.op.	+	i	i	i	0	0	0
R 0·2	sl.op.	i	i	i	i	0	0	0
H 0·2	sl.op.	+	+	+	+	+	+	+
NaCl								
R 0·006	clear	+	+	+	0	0	0	0
R 0·012	clear	+	+	i	i	0	0	0
R 0·025	sl.op.	+	i	i	i	i	0	0
R 0·05	sl.op.	i	i	i	i	i	i	0
R 0·2	sl.op.	i	i	i	i	i	i	0
R 0·3	opal.	i	i	i	i	+	+	+
R 0·6	st.op.	−	−	+	+	+	+	+
H 0·2	sl.op.	+	+	+	+	+	+	+
Na_2SO_4								
R 0·003	clear	+	+	+	+	+	+	+
R 0·006	sl.op.	+	+	i	i	0	0	0
R 0·012	opal.	i	i	i	i	i	0	0
R 0·025	pptt.	.	.	.	.	.	.	.
H 0·012	sl.op.	+	+	+	+	+	+	+

Precipitin test. 1 ml. of an antiserum to human albumin at a dilution 1/40 was added to 1 ml. of antigen solutions at various concentrations. + indicates precipitation and − no precipitation if test for inhibition was not made. Inhibition was tested by addition of 0·1 ml. of 0·03% solution of unheated human albumin to the mixtures where there was no precipitation. i indicates inhibition, 0 indicates no inhibition.

The solutions were heated for 10 min. at 83°.

R, heated solution contained 0·05% of human and 0·5% of rabbit albumin.

H, heated solution contained 0·05% of human albumin.

sl.op. slight opalescence; opal. moderate opalescence; st.op. strong opalescence; pptt. precipitation.

antigens, have been used as a test for their formation [cf. Bawden & Kleczkowski, 1941].

The formation of complexes between albumin from human serum and albumin from rabbit serum was studied, using an antiserum to human albumin. Dialysed solutions of the two albumins were adjusted to *p*H 7·0 and *p*H 3·2 by adding 0·1 *N* NaOH or HCl respectively. They were then heated separately and in mixtures containing ten times as much rabbit albumin as human albumin. They were heated for 10 min. at 83° salt-free and in the presence of various salts at different concentrations. After cooling, the solutions at *p*H 3·2 were adjusted to *p*H 7·0 by adding 0·1 *N* NaOH. All the heated solutions were diluted in physiological saline and tested serologically for their ability to give precipitation or inhibition with human albumin antiserum. The results are given in Table 2.

Human albumin heated separately at either *p*H 7·0 or 3·2, salt-free, or in the presence of all the tested salts, precipitated with the antiserum to human albumin. This specific precipitation was not affected if rabbit albumin, either unheated or heated separately, was mixed with the heated human albumin. The salt-free solutions of the mixtures of human and rabbit albumins, heated at either *p*H, also precipitated with the antiserum. Heating these mixtures in the presence of all the salts, on the other hand, produced non-precipitating complexes that inhibited the precipitation of human albumin added subsequently.

For each salt four zones of concentration can be distinguished, in each of which the effect of heating the protein mixture is different. At low concentrations no serologically demonstrable complex is formed and heated solutions are precipitated by the antiserum. In the next zone a serologically non-precipitating complex is formed. Heating in the third zone gives material that is precipitated specifically by the antiserum. This material flocculates with antiserum more quickly than the unheated human albumin and forms larger floccules. In the zone of highest salt concentration a precipitate of denatured protein separates during heating.

Hardy [1900] showed that the efficiency of salts in flocculating colloidal solutions (including proteins) is determined by the valency of the ion with the charge opposite to that of the colloidal particles. The efficiency is expressed as the reciprocal of the lowest concentration, in terms of normality, necessary to cause flocculation. Ions of higher valency are much more efficient than those of lower valency. Chick & Martin [1912] found that Hardy's law also applies to the efficiency of salts in flocculating protein solutions previously denatured by heat in the absence of salts. The results given in Table 2 show that this law also applies to the formation of complexes between two different proteins. At *p*H 7·0, i.e. on the alkaline side of the isoelectric point of heated proteins, when the protein particles are negatively charged, the efficiency of salts in causing the complex formation is determined by the valency of the cations. The lowest concentration of $Al_2(SO_4)_3$ causing the complex formation was approximately one-tenth of that of $MgSO_4$ or $BaCl_2$, and this was approximately one-tenth of that of Na_2SO_4 or NaCl. At *p*H 3·2, i.e. on the acid side of the isoelectric point of the heated proteins, when the protein particles are positively charged, the efficiency of salts in causing the complex formation depends on the valency of the anions. The lowest concentration of Na_2SO_4 causing the complex formation was approximately one-tenth of that of $BaCl_2$.

The fact that the efficiency of salts in promoting the complex formation is determined by the valency of the ion charged oppositely to the protein particles, strongly suggests that the action of salts is electrostatic. They can be pictured as acting by discharging the protein particles, thus facilitating their combination with each other, as when they cause flocculation of colloidal suspensions. The formation of protein complexes, however, is not altogether comparable with flocculation. Flocculation is caused whether salts are present during the heating or are added to the protein solutions after heating. The formation of protein complexes, however, takes place to any appreciable extent only when the mixtures are heated in the presence of salt. If salt-free mixtures of human and rabbit serum-albumin fractions are heated and salt added after cooling, no material that inhibits serological precipitation can be detected, and the heated mixture precipitates with the antiserum to human albumin in exactly the same way as human albumin heated alone.

The effect of salt on the formation of a serologically non-precipitating complex between tomato bushy-stunt virus and rabbit serum albumin during heating

Table 3 shows the influence of NaCl on the formation of serologically non-precipitating complex between tomato bushy-stunt virus and rabbit-serum albumin. In salt-free solution no demonstrable amount of the complex was formed, and the heated virus-albumin mixture behaved serologically like a solution of the virus heated alone. When heated in the presence of salt, a non-precipitating complex was formed, and the lowest concentration of NaCl causing the complex formation was approximately equal to the lowest NaCl concentration causing formation of the complex between human- and rabbit-serum albumins (cf. Table 2).

With this system also, the addition of salt to the virus-albumin mixture previously heated salt-free did not cause the formation of any demonstrable quantities of a non-precipitating complex.

Table 3. *The effect of NaCl on the formation of non-precipitating complexes between tomato bushy-stunt virus and rabbit serum albumin*

Concentration of NaCl (normality) in heated solutions	Appearance of the solutions after heating	Precipitin test Dilutions of the antiserum 1/20	1/40	1/80	1/160	1/320	1/640
Unheated bushy-stunt virus		+	+	+	+	+	–
Heated virus-albumin mixture:							
0·012	clear	+	+	+	+	–	–
0·025	sl.op.	0	0	0	i	i	–
0·05	sl.op.	0	i	i	i	i	–
0·1	opal.	i	i	i	i	i	–
Salt-free	clear	+	+	+	+	+	+
The virus heated alone:							
0·1	st.op.	+	+	+	+	+	+

Precipitin test. 1 ml. of every antigen preparation diluted 1/10 in physiological saline was mixed with 1 ml. of an antiserum to bushy-stunt virus at varying dilutions.
Inhibition was tested by adding 0·1 ml. of 0·05% solution of the unheated virus.
The solutions were heated for 10 min. at 83° at *p*H 7·0.
Virus-albumin mixture contained 0·05% of bushy-stunt virus and 0·5% of rabbit serum albumin.
The virus alone was used as 0·05% solution.
The symbols as in Table 2.

The effect of salt on the formation of a serologically non-precipitating complex between soluble human serum globulin and rabbit serum albumin during heating

Table 4 shows that when the water-soluble fraction of human-serum globulin was heated with rabbit-serum albumin, rather different results were obtained. In the presence of salt the system resembled the other two described above; a serologically non-precipitating complex was formed which inhibited the precipitation of the unheated globulin added later. The mixture heated in the absence of salt, however, also failed to precipitate with the antiserum, although it did not give the phenomenon of inhibition. When the globulin was heated alone, it still precipitated specifically with the antiserum whether rabbit albumin was later added or not. Thus the change in behaviour of the salt-free mixture was a result of an interaction between the two proteins during heating.

It has been shown above, by measuring changes in the precipitability with $MgSO_4$, that there was some combination between rabbit euglobulin and albumin when a mixture was heated salt-free, although much less than when heated in the presence of NaCl. The results in Table 4 suggest that a complex was also formed between human globulin and rabbit albumin when heated salt-free. It appears that the complex was formed in an amount sufficient to destroy serological precipitability but not sufficient to produce the inhibition phenomenon, which was well pronounced when NaCl was present during heating and the complex formation occurred to a greater extent.

Table 4. *The effect of NaCl on the formation of non-precipitating complexes between soluble human serum globulin and rabbit serum albumin*

Treatment of human globulin	Appearance of the solutions after heating	Precipitin test Human globulin mg. 0·5	0·25	0·12	0·06	0·03	0·015	0·0075
Unheated		+	+	+	+	+	+	+
Heated with albumin in 1% NaCl	st.op.	i	i	i	i	0	0	0
Heated with albumin salt-free	clear	0	0	0	0	0	0	0
Heated alone in 1% NaCl	pptt.	.	.	.	.	.	.	.
Heated alone salt-free	st.op.	+	+	+	+	–	–	–

Precipitin test. 1 ml. of an antiserum to human globulin at a dilution 1/10 was added to 1 ml. of antigen solutions at various concentrations.
Inhibition was tested by adding 0·1 ml. of 0·06% solution of unheated soluble human globulin.
The solutions were heated for 10 min. at 83° at *p*H 7·0.
In all heated solutions concentration of human globulin was 0·05% and that of rabbit albumin 0·5%.
The symbols as in Table 2.

The effect of salt on the formation of non-precipitating complexes between antibodies and unspecific serum proteins during heating

Antibodies can undergo initial stages of heat denaturation without losing their ability to combine with their antigens, and, like protein-antigens, when heated in the presence of other proteins undergoing denaturation simultaneously, they can combine with them to form complexes [Kleczkowski, 1941*b*]. If combined with sufficient albumin, they do not precipitate their antigens, but, as they still combine with them, they interfere with the action of precipitating antibodies. The degree of this interference depends on the nature of antigen, being large with 'O'-type antigens and small with 'H'-type antigens. Heating whole antisera can lead to the formation of such complexes produced by heat-changed antibody particles combining with serologically unspecific proteins present in the sera.

To test the effect of salt on the formation of such antibody complexes, dialysed antisera to human-serum albumin and to tomato bushy-stunt virus were used. The precipitate of insoluble globulin formed during dialysis was filtered off and the filtrate was used as test solution. This involved loss of antibodies, but sufficient remained in solution for the test. The results of heating the dialysed antisera with and without addition of NaCl are given in Table 5.

DISCUSSION

All the antigens used in this work belong to the group of proteins which, when heated in the presence of salt with serologically unspecific serum albumin, combine with it to form serologically non-precipitating complexes. In spite of their uniform behaviour in the presence of salt, when heated salt-free they behave differently. Bushy-stunt virus, and human-serum albumin, heated with rabbit-serum albumin in salt-free solutions did not form any demonstrable amounts of non-precipitating complexes; they still precipitated with their antisera exactly as if they had been heated alone. On the other hand, the water-soluble fraction of human-serum globulin heated salt-free in the presence of rabbit-serum albumin formed a non-precipitating complex, although to a smaller extent than in the presence of salt. This complex did not precipitate with the antiserum, but it differed from that formed in the presence of salt as it did not show the presence of inhibition. It is difficult to account for these differences satisfactorily, although various tentative explanations can be offered. Susceptibility to the presence of traces of salts, which might have remained after dialysis, may vary with different proteins, so that complex formation could proceed in globulin-albumin mixtures but not in the two other protein systems. The differences may be apparent rather than real, for only the precipitin test was used to detect the formation of the complexes. Therefore, if the different antigens vary in their sensitivity to the presence of the complexes, they may have been formed equally in all three systems in the absence of salts, but only with globulin may it have been sufficient to prevent precipitation. It is, however, equally possible that the globulin differs from albumin and the virus when heated in the absence of salt, and that the first can combine with rabbit-serum

Table 5. *The effect of NaCl on the formation of non-precipitating complexes between antibodies and unspecific serum proteins*

	The antiserum to human albumin Human albumin (mg.)						The antiserum to bushy-stunt virus Dilutions of the antiserum					
Treatment of the antisera	0·25	0·12	0·06	0·03	0·015	0·0075	1/10	1/20	1/40	1/80	1/160	1/320
Unheated	+	+	+	+	+	+	+	+	+	+	–	–
Heated in 1% NaCl	0	0	i	i	i	i	i	i	i	i	0	0
Heated salt-free	+	+	+	+	+	+	i	i	i	0	0	0

Dialysed antisera were heated for 10 min. at 80° at *p*H 7·0 at a dilution 1/10 in distilled water with and without addition of 1% NaCl.

Precipitin test. The antiserum to human albumin was tested by mixing 1 ml. of the antiserum diluted 1/10 with 1 ml. of solutions of human albumin at various concentrations. The inhibition was tested by adding 0·1 ml. of undiluted dialysed antiserum.

The antiserum to bushy-stunt virus was tested by mixing 1 ml. of 0·005% solution of the virus with 1 ml. of various dilutions of the antiserum. The inhibition was tested by adding 0·1 ml. of the dialysed antiserum diluted 1/2.

The symbols as in Table 2.

Both antisera behaved similarly when heated in the presence of salt, forming non-precipitating and inhibiting complexes. They showed, however, differences after heating salt-free. The antiserum to human albumin still precipitated its antigen, whereas the antiserum to bushy-stunt virus did not precipitate and instead gave inhibition, although the 'inhibition titre' was slightly lower than that given by the same antiserum after heating in the presence of salt.

albumin in these conditions whereas the others cannot.

Whether or not the presence of salt is necessary to start the combination of two proteins, it certainly greatly increases it. As in causing flocculation, the efficiency of different salts in influencing complex formation depends on the valency of the ion with the charge opposite to that of the protein particles. The two processes, however, are not identical. If the protein is denatured by heating in the absence of salts, the subsequent addition of salt causes flocculation. By contrast, salts influence complex formation only if they are present in protein mixtures during heating, for the addition of salt to a protein mixture heated salt-free does not lead to formation of any measurable amount of serologically non-precipitating complexes. This difference could be explained by assuming that individual proteins differ in their rates of aggregation, and that these rates are much greater than rates of denaturation. Then, if salt is present during heating, particles of different proteins would be able to combine with one another as soon as they are denatured, but if salt is added to a mixture of proteins already denatured by heating in salt-free solution, each protein would tend to aggregate separately.

The results with antibodies almost exactly parallel those with antigens. The two antisera used were to bushy-stunt virus and to human-serum albumin. When heated in the presence of salt, these behave similarly, the antibodies forming non-precipitating complexes which inhibit precipitation. In the absence of salts the two antisera behaved differently. The virus antiserum again ceased to precipitate and instead inhibited precipitation, though less strongly than when heated with salt, whereas the antiserum to human albumin still precipitated. This difference at first sight suggests differences in the heat stabilities of the two antisera. A similar difference between the behaviour of antisera to 'O' and 'H'-type antigens is well known and was for long interpreted as evidence that these antigens produced antibodies with different properties. It has now been shown, however, that antibodies to antigens of both types undergo similar changes when heated, and that the difference in their behaviour occurs because the precipitability of the antigens is affected differently by the presence of antibodies changed into non-precipitating complexes [Kleczkowski, 1941*b*; Bawden & Kleczkowski, 1941, 1942]. Similarly, the difference between the behaviour of dialysed antisera to human albumin and to bushy-stunt virus respectively, may be because the two antigens are affected differently by the presence of non-precipitating antibody complexes, which are probably formed to a small extent when both antisera are heated in the absence of salts.

SUMMARY

The formation of complexes between different proteins, undergoing heat denaturation together, is greatly influenced by the presence of salts. In the absence of salts only mixtures containing water-soluble serum globulin formed any detectable amount of complexes.

The efficiency of different salts in promoting the complex formation, expressed as the reciprocal of the lowest effective concentration, follows Hardy's law. Ions of higher valency are much more effective than those of lower valency. On the alkaline side of the isoelectric point of the proteins, the efficiency of salts is determined by the valency of the cations, and on the acid side by the valency of anions.

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The Influence of Halide Concentration on the Metabolism of *Penicillium sclerotiorum* van Beyma

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In a previous communication [Curtin & Reilly, 1940], the isolation and properties of sclerotiorine, a chlorine-containing metabolic product of *Penicillium sclerotiorum* van Beyma, were described. Reference was also made to a second product, a red pigment retained by the mother-liquors of crystallization. Work on the constitution of sclerotiorine was somewhat hampered by the small yield, 2% of

the dry mycelium weight. The possibilities of increasing this figure by alteration of the chloride concentration and by activation with yeast extract [Abraham, Chain, Fletcher, Florey, Gardner, Heatley & Jennings, 1941] have now been investigated, the basal medium being that of Czapek-Dox. Simultaneously, the organism has been grown on media devoid of chlorides, viz. Raulin-Thom and Raulin-Dierckx media together with a Czapek-Dox medium compounded without KCl, in the hope of obtaining the red component in a more workable form. The mycelium pigmented in all cases, and the dark red product of light petroleum extraction was submitted to diverse processes to free it from the fats and other products of the extraction. Sublimation in a high vacuum, separation by adsorption on Al_2O_3 and $Ca(OH)_2$, and crystallizations at the interface of two immiscible liquids gave no substance that could be isolated other than as a glaze on glass.

Inoculations of the mould on Czapek-Dox media, containing bromides or iodides in amounts equivalent to the usual KCl concentration, gave no crystalline bromo- or iodo-derivatives. This finds a parallel in the work of Raistrick & Smith [1936]. However, appreciable darkening in the colour of the mycelium and its extract was noted with each change of halide.

Cultural conditions. The culture used throughout was the 6th transfer from that obtained in March 1940 from the Centraalbureau voor Schimmelcultures, Holland. Sucrose was used as the source of carbon, trial cultures proving that this sugar was satisfactory.

The substrate employed was the acid Czapek-Dox medium (sucrose, 50 g.; $NaNO_3$, 2 g.; KH_2PO_4, 1 g.; KCl, modified; $MgSO_4$, $7H_2O$, 0·5 g.; $FeSO_4$, $7H_2O$, 0·01 g.; distilled water to 1000 ml.). Four batches, each of 5 l., containing 0·25, 0·5, 0·75 and 1·0 g. KCl/l., respectively as the sole source of chlorine were prepared. The media were distributed in 100 ml. lots in 20 oz. flat bottles, the cotton-wool plugs being paper-capped to prevent dilution of the medium during sterilization by steaming on each of three consecutive days. The bottles were inoculated with a spore suspension from a 14-day-old culture of *P. sclerotiorum* on malt agar, and incubated at 25°. Three bottles were filtered at the pump every 3 days, and the filtrate tested for *p*H, sugar concentration by polarimeter and for residual KCl.

In all inoculations described, the *p*H value reached a minimum on the 9th day and reverted to approximate neutrality on the 24th day, in agreement with the figures already published [Curtin & Reilly, 1940]. The production of sclerotiorine was thus uninfluenced by the acidity of the medium. The polarimeter readings were of particular interest. Due to their similarity, one series will suffice as an illustration (see Table 1).

Table 1

Days of incubation	Polarimeter reading
0	5·7° D
3	2·6° D
6	0·3° L
9	1·0° L
12	0·6° L
15	0·0
18	0·0
21	0·0
24	0·0

The connexion between the minimum *p*H value on the 9th day and the maximum *laevo* reading is obvious. The main interest centres round the fact that more than four-fifths of the sucrose was metabolized in so short a period as 9 days.

Estimation of residual chloride. Direct titration was not possible as the medium reduces both $KMnO_4$ and $AgNO_3$. Oxidation by refluxing with conc. HNO_3 was unsatisfactory. A modified form of the method described by Raistrick & Smith [1936] was finally employed:

50 ml. of the filtered medium were shaken for 10 min. with 2·5 g. of norit charcoal and filtered at the pump without washing. 25 ml. of this filtrate were taken and excess $AgNO_3$ added, together with 5 ml. of 6*N* HNO_3, which had been boiled free from HNO_2. The precipitated AgCl was coagulated by boiling, filtered off and washed with a little dilute HNO_3. On cooling below 25°, the new filtrate was titrated against *N*/100 NH_4CNS, using 1 ml. of standard Volhard indicator.

In actual practice, the medium extracts chlorides from the norit, the KCl equivalent, as determined by a series of blanks, being 0·00095 g./25 ml. of the medium. The residual percentage KCl, at intervals, over a period of 24 days is shown in Table 2.

Table 2

Days of incubation	Initial chloride content of media: 0·25 g. KCl/l.	0·5 g. KCl/l.	0·75 g. KCl/l.	1·0 g. KCl/l.
	Residual chloride content			
	%	%	%	%
0	0·0250	0·0497	0·0747	0·0994
3	0·0172	0·0369	0·0527	0·0762
6	0·0071	0·0274	0·0509	0·0735
9	0·0050	0·0258	0·0499	0·0729
12	0·0070 (?)	0·0250	0·0473	0·0705
15	0·0033	0·0250	0·0470	0·0678
18	0·0027	0·0235	0·0437	0·0661
21	0·0015	0·0233	0·0457 (?)	0·0640
24	0·0009	0·0230	0·0475 (?)	0·0640
Metabolized KCl	0·0241%	0·0267%	0·0310%	0·0354%

Plotting the residual KCl against time gives four curves of identical shape, which show that the utilization of KCl becomes regular after 9 days. Before this, the halide is rapidly taken up. We have

already stressed the rapidity with which the sugar is utilized during this same period.

The filtered mycelium from 25 bottles (2·5 l.) of each of the four media was pressed, vacuum-dried, and extracted with light petroleum. A mean value of 12·9 g. dry mycelium/l. was taken as the basis of calculation. The extracted sclerotiorine was weighed on recrystallization, the yields of pigment being practically constant, allowing for the necessarily incomplete separation from the solvent (see Table 3, col. 2).

Completion of the chlorine balance sheet was effected by the estimation, as KCl, of the chloride content of the extracted mycelium. The method [Schiff, 1879; 1906] was as follows:

Mycelium (2–3 g.) was weighed into a small platinum crucible and covered with an intimate mixture of 1 part pure Na_2CO_3 and 4 parts pure CaO and inverted into a larger crucible which was then completely filled with the same mixture. The whole was then heated with a blow-pipe so that the outer parts attained a high temperature before the mycelium began to decompose. After heating to redness and cooling, the mixture was treated with distilled water, the solution being acidified with pure HNO_3, an appreciable rise in temperature being avoided. The solution was then adjusted to known volume and the chlorides estimated by the Volhard method already described.

The metabolized KCl and the KCl equivalents of sclerotiorine and the mycelia are given in Table 3. The 5th column, KCl unaccounted for, shows the quantities of KCl retained by the mother-liquors of crystallization, its presence having been duly verified.

Table 3

KCl/l. of medium g.	Yield of sclerotiorine g./l. medium	KCl metabolized as pigment g./100 ml. medium	KCl as mycelium %	KCl unaccounted for %
0·25	0·190	0·0038	0·0127	0·0076
0·50	0·213	0·0042	0·0118	0·0107
0·75	0·189	0·0038	0·0128	0·0144
1·00	0·220	0·0044	0·0136	0·0174

The results so far may be summarized as follows: Yields of pigment and the chlorine content of the mycelium are, within the limits of experimental error, practically constant, while the chlorine content of the mother-liquors are in direct proportion to the KCl concentration of the medium.

Effect of pH and addition of yeast extract. A further series of inoculations to test the influence of *p*H and yeast extract on the rate of KCl metabolism was now completed, *p*H, polarimeter and Volhard titrations being performed as already described. For the influence of *p*H, 5 l. of neutral Czapek-Dox medium were made up by K_2HPO_4 instead of KH_2PO_4 as in the acid medium (*p*H 4·2).

Yeast extract was prepared by macerating 500 g. pressed yeast with 1 % soda solution, re-pressing on the Buchner funnel and washing with distilled water. The yeast, suspended in 1 l. of distilled water, was boiled for 1 hr. and allowed to stand overnight, when it was centrifuged and the volume of the clarified extract adjusted to 1000 ml. The extract was then sterilized by steaming on three consecutive days. Two batches of 5 l. standard Czapek-Dox medium were then made up, incorporating 5 and 10 % by volume respectively, of the yeast-water sown and incubated as above. All three media showed the same rapid elimination of sugar, but the *p*H of the neutral medium, on reaching its minimum value, displayed only a slight tendency to revert to neutrality. In estimating the KCl content of the yeast-extract media, it was found necessary to add an additional blank of 0·00044 g. for each 5 % of yeast water. The corrected KCl percentages are shown in Table 4.

Table 4

	Media		
Days of incubation	Neutral Czapek-Dox	Neutral Czapek-Dox plus 5 % yeast extract	Neutral Czapek-Dox plus 10 % yeast extract
	% residual KCl content of media (corrected)		
0	0·0497	0·0500	0·0496
3	0·0375	0·0434	0·0482
6	0·0354	0·0422	0·0452
9	0·0339	0·0405	0·0434
12	0·0315	0·0400	0·0425
15	0·0309	0·0350	0·0375
18	0·0309	0·0200	0·0202
21	0·0295	0·0154	0·0200
24	0·0250	0·0140	0·0173
Metabolized KCl	0·0247 %	0·0360 %	0·0323 %

The disappearance of KCl in the neutral medium is slower though more regular than in the acid medium. The yeast water decelerates the initial KCl metabolism up to the 12th day, after which the halide content drops rapidly until the 15th day. It seems reasonable therefore to assume that the activating effect of yeast extract is first directed towards the sugar metabolism to the detriment of the chloride, but that when the sugar has been eliminated, the activation is completely centred round the utilization of the KCl present.

The decreased rate of KCl metabolism during the early stages of growth definitely reduces the yield of pigment. In all three of the above inoculations, the amounts of recrystallized sclerotiorine obtained were only slightly in excess of 1 % of the dry mycelium weight.

SUMMARY

Growth of *P. sclerotiorum* on media containing KBr and KI in place of the usual KCl did not yield halide derivatives analogous to sclerotiorine, the ordinary

chlorinated metabolic product, while growth on media devoid of chloride in no way assisted the isolation of the red component. Variation of the KCl concentration showed that its elimination from the medium was quite regular after the initial period when the sugar metabolism was exceptionally rapid. Increased chloride concentration had no effect on the production of sclerotiorine and only affected the chlorine content of the other products of extraction of the mycelium. It is suggested that the pigment is a metabolic by-product rather than the fundamental end-product of the chloride metabolism. The activating effects of yeast extract was no more than equivalent to the effect of increased KCl concentration.

The authors desire to thank Prof. J. Reilly for his continued interest during the progress of this work.

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The Determination of 2:3-Diketo-*l*-gulonic Acid

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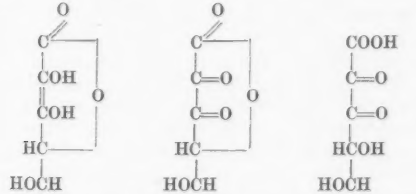
The first stage of the oxidation of *l*-ascorbic acid is of particular interest to the biochemist. The product, dehydro-*l*-ascorbic acid, retains its lactone ring and can be reduced to *l*-ascorbic acid by mild reagents such as H_2S, etc., in vitro, and can also be regenerated in vivo almost quantitatively. In aqueous solutions, dehydro-*l*-ascorbic acid undergoes mutarotation which is generally assumed to be due to the gradual opening of its lactone ring with the formation of 2:3-diketo-*l*-gulonic acid. This latter compound can still be converted into *l*-ascorbic acid by lactonization and reduction when treated with HI in vitro [Herbert, Hirst, Percival, Reynolds & Smith, 1933]. Regeneration apparently does not occur in vivo, since little if any antiscorbutic activity has been observed in solutions of dehydro-*l*-ascorbic acid which have undergone mutarotation for some time [Hirst & Zilva, 1933; Borsook, Davenport, Jeffreys & Warner, 1937].



l-Ascorbic acid Dehydro-*l*-ascorbic acid 2:3-Diketo-*l*-gulonic acid

Whilst dehydroascorbic acid can easily be determined by titration of the regenerated ascorbic acid after reduction with H_2S, no direct method is available for the determination of diketogulonic acid. In this communication conditions are described under which this compound, which so far has not been isolated, can be determined directly.

Herbert *et al.* [1933] have shown that both ascorbic acid and freshly oxidized ascorbic acid give derivatives with 2:4-dinitrophenylhydrazine of m.p. 282° (decomp.) and 280° (decomp.) respectively. We have found that diketogulonic acid obtained by mutarotation from dehydroascorbic acid also gives with 2:4-dinitrophenylhydrazine a red derivative or derivatives which on solution in NaOH produce an intensely red colour. This colour reaction forms the foundation on which the procedure for the direct determination of the diketogulonic acid is based.

2:4-Dinitrophenylhydrazine reacts also with phenylpyruvic acid [Penrose & Quastel, 1937] and with other α-keto acids [Lu, 1939; Sealock & Silberstein, 1940; Sealock & Scherp, 1941], and consequently the reaction cannot be considered specific. In actual practice, however, it has been found that extracts of the tissues of normal guinea-pigs, and untreated human urine give a very low and fairly constant colour with dinitrophenylhydrazine; thus it should be possible to detect the appearance of even small quantities of diketogulonic acid by this reaction. Furthermore, the results suggest that the procedure outlined by us is open to further improve-

ments as regards specificity and sensitivity should the necessity arise.

GENERAL TECHNIQUE

Reagents. (1) 2 g. 2:4-dinitrophenylhydrazine (laboratory reagent) were dissolved with warming in 1 l. 2*N* HCl (AnalaR). The stock solution was filtered before use.

(2) *N* NaOH (AnalaR).

(3) *l*-Ascorbic acid, 99–100 % purity. Messrs Roche Products, Ltd.

(4) Dehydro-*l*-ascorbic acid was obtained by oxidizing *l*-ascorbic acid with the theoretical amount of 0·1*N* I_2 (AnalaR). Yield as determined by reduction with H_2S, 95–100 %. The solutions were used immediately after preparation.

(5) 2:3-Diketo-*l*-gulonic acid was prepared by allowing a solution of dehydro-*l*-ascorbic acid to mutarotate for 18 days at room temperature without neutralizing the HI formed in the preparation of the dehydroascorbic acid [cf. Herbert *et al.* 1933]. After this time the ascorbic acid regenerated from the solution by reduction with H_2S reached a value varying from 10 to 15 % of its original concentration, and this remained approximately constant for at least 10 days. The intensity of the colour given by the solutions with 2:4-dinitrophenylhydrazine increased during mutarotation and also became constant after about 18 days. Diketogulonic acid more than 10 days old was never used in this investigation.

Factors influencing the reaction. The intensity of the colour produced could be increased by raising the quantity of dinitrophenylhydrazine used in the reaction. The amount of the reagent that could be employed was, however, limited by the fact that when certain quantities of the hydrazine were exceeded the addition of the alkali made the reacting solutions turbid. For the range of concentrations of diketogulonic acid used by us the addition of 1 ml. of the hydrazine solution produced a colour of suitable intensity without influencing the transparency of the solution. The use of 2:4-dinitrophenylhydrazine (laboratory reagent) recrystallized from ethyl acetate did not influence the colour reaction in any way. The time and temperature of heating of a mixture of diketogulonic, dehydroascorbic, and ascorbic acids with dinitrophenylhydrazine greatly affected the intensity of the colour; this will be discussed in detail later.

As would be expected the quantity of NaOH which it was necessary to add depended on the quantity of acid present in the reacting solution, but under the above conditions 4 ml. of *N* NaOH amply satisfied the requirement, and an increase to 6 ml. was without influence on the colour production.

After the addition of the *N* NaOH a brown colour developed immediately, which rapidly turned to orange in dilute and red in more concentrated solutions. As the final colour is unstable it was necessary to measure its intensity at a given time soon after production.

Measurement of colour. The measurement was carried out in a Duboscq type of colorimeter. Owing to the instability of the colour it was, however, more convenient to have recourse to a photometric technique [King & Haslewood, 1937] instead of matching the colour directly. For this purpose a spectrum green colour filter (Ilford no. 604) was introduced into the eyepiece of the instrument and the standard was replaced by neutral density filters against which the absorption was measured. Although a range of these filters was employed all results were calculated for the 0·5 neutral density filter.

Effect of time and temperature on colour production. As already mentioned the time and temperature of heating diketogulonic, dehydroascorbic or ascorbic acids with dinitrophenylhydrazine greatly influenced the intensity of the colour production. This is probably due in part to the changes the last two compounds undergo to yield dinitrophenylhydrazine derivatives similar to, if not identical with, the derivative yielded by diketogulonic acid. The reaction was therefore studied after various periods of heating at 25°, 50° and 75°. To each of a series of tubes containing 9 ml. solution of the individual acids previously brought rapidly to about 2° above the desired temperature, 1 ml. of dinitrophenylhydrazine reagent was added and the tubes placed in the respective thermostats. Those mixtures which were submitted to high temperatures were lightly corked to avoid evaporation. The solutions were then shaken for a short time in order to adjust their temperature to that of the thermostat. At various intervals one of each of the series was withdrawn, cooled rapidly if necessary, treated with the alkali, diluted to 25 ml., and the colour measured as described above.

The results are given in Figs. 1, 2 and 3. On careful examination of these data it appeared to us that the colour produced after 1 hr. at 25° was the most suitable for the quantitative assessment of diketogulonic acid in the presence of the other two acids, for the following reasons. It will be seen from Fig. 1 that the intensity of the colour given by dehydroascorbic acid under these circumstances is only about 8 % of that given by its equivalent of diketogulonic acid and that this percentage increased with time. At 50° and 75° (Figs. 2 and 3) on the other hand the reaction of dehydroascorbic acid is increased in 1 hr. to as much as 44 % and 81 % respectively of that of its equivalent of diketogulonic acid. Furthermore at 25° ascorbic acid even in quantities as high as 2 mg. does not react at all. In addition it might be mentioned that as the reaction proceeded there was a tendency for the formation of a reddish precipitate, especially at the higher temperatures, which was

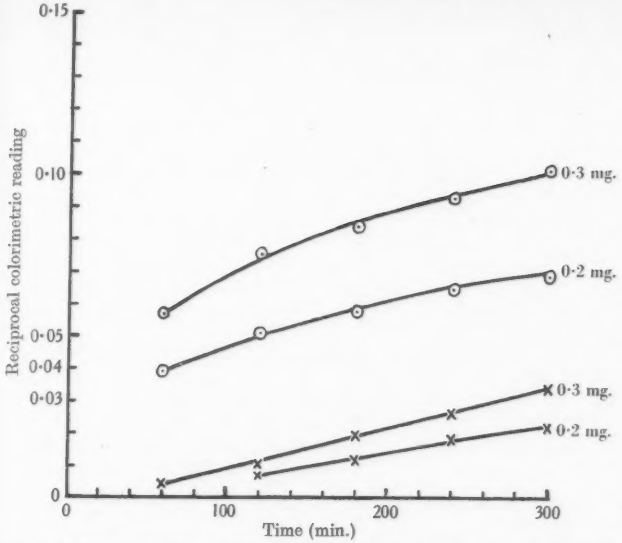


Fig. 1. Colour production at 25°. ⊙—⊙ diketogulonic acid. ×—× dehydroascorbic acid. In this and the following graphs, diketogulonic and dehydroascorbic acids are expressed as equivalent quantities in mg. of ascorbic acid from which they were prepared.

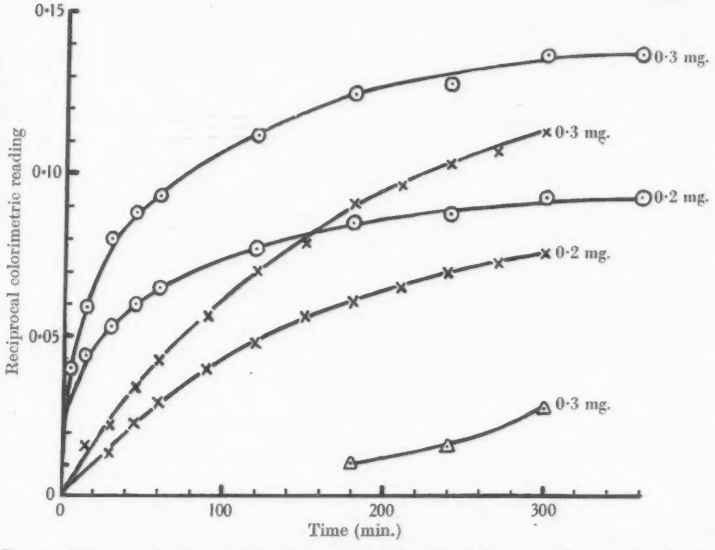


Fig. 2. Colour production at 50°. △—△ ascorbic acid. Other symbols are as in Fig. 1.

soluble in *N* NaOH. At 25° the amount of this precipitate was very small. It is also of significance that in all these experiments the colour produced at any time when expressed as a percentage of the maximum colour obtained at 75° after 300 min., was the same in the two concentrations both for dehydroascorbic acid and diketogulonic acid. This was also found to be true for ascorbic acid when it was possible to obtain significant figures namely, at 75°.

Calibration curve. Calibration curves for diketogulonic acid and dehydroascorbic acid covering a range of 0·05–0·5 mg. in the case of the former and 0·35–0·5 mg. in that of the latter, were constructed from the figures obtained by keeping the reaction mixtures for 1 hr. at 25°. For convenience the diketogulonic acid is expressed as mg. of ascorbic acid from which it was prepared, since an absolute value cannot be obtained owing to the fact that it has not been isolated. These units are plotted against the reciprocal of the reading on the colorimeter scale ($1/R$). The results are the mean of the figures obtained from three different solutions determined in duplicate; the variation of the individual readings from the mean were within the limits of ±3%. As can be seen from the curve (Fig. 4) the relationship between the concentration and the reciprocal colorimeter readings is linear up to about 0·4 mg. of diketogulonic acid; beyond that concentration there is a slight deviation from Beer's Law. Since the intensity of the colour produced by dehydroascorbic acid is only about 1/12 of that produced by diketogulonic acid it was not possible to obtain values lower than 0·35 mg. It is seen that figures for the colour given by diketogulonic acid in pure solution approximating concentrations as low as 1 part in 200,000 can be read off the calibration curve.

The curve does not pass through the origin. This can be explained in two ways. Either some absorp-

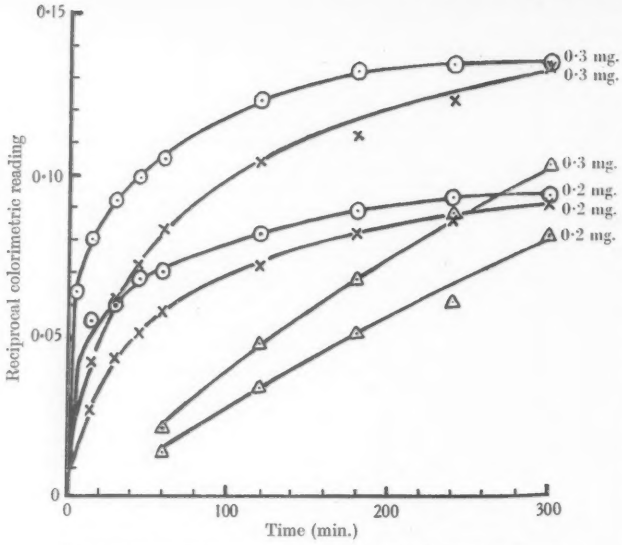


Fig. 3. Colour production at 75°. The symbols are as in Fig. 2.

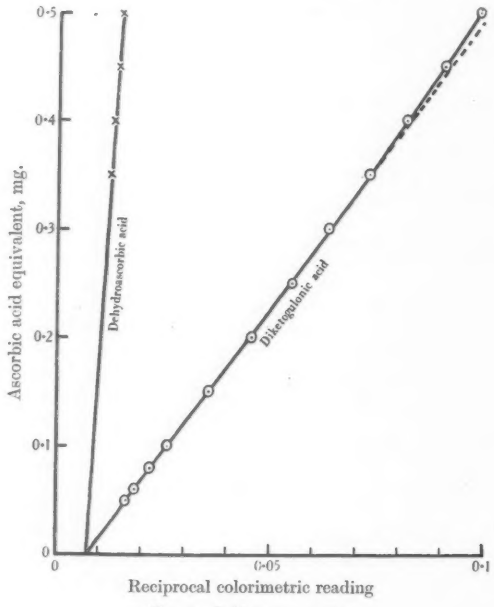


Fig. 4. Calibration curve.

tion is produced by the reagents or Beer's Law is not obeyed in very dilute solution. We favour the former explanation, since some experimental evidence was obtained supporting this view. By measuring the absorption of solutions of $KMnO_4$ with the spectrum green filter, a linear relationship between $1/R$ and the concentration of the $KMnO_4$ could be obtained, obeying Beer's Law even in dilute solution, i.e. the curve passing through the origin. When a blank solution containing 1 ml. of dinitrophenylhydrazine and 4 ml. *N* NaOH in 25 ml. was matched against a suitable dilution of $KMnO_4$ it was found that the value for the absorption of the concentration of $KMnO_4$ equivalent to the blank expressed in units of the 0·5 neutral density filter coincided with the zero value obtained from the calibration curve (0·007). All results including those given in Figs. 1, 2 and 3 were therefore corrected for zero displacement.

Procedure. A quantity of the solution containing 0·05–0·5 mg. diketogulonic acid is introduced into a 6 × 1 in. tube and the volume, if necessary, is made up to 9 ml. with distilled water. 1 ml. of the dinitrophenylhydrazine reagent is then added and the tube placed in a thermostat at 25° (± 0·2°) for 1 hr. At the end of this time 4 ml. of *N* NaOH are added, the solution is transferred to a 25 ml. graduated flask and after 1 min. is made up to volume with the washings and with distilled water. After mixing the contents the colour of the solution is measured in the colorimeter as rapidly as possible (30 sec.) in order to avoid any error due to fading. Six readings are taken which should not deviate by more than ± 2 % from the mean. The quantity of diketogulonic acid present is then read off the calibration curve.

If dehydroascorbic acid is present it is determined by reduction with H_2S and titration with indophenol and the colorimetric equivalent is obtained from the calibration curve. The calculation is then made as follows:

$$\frac{1}{R_y}=\frac{1}{R}-\frac{1}{R_x}+\frac{1}{R_c}.$$

R = Observed colorimetric reading.
R_x = Equivalent colorimetric reading for quantity of dehydroascorbic acid found.
R_c = Colorimetric reading due to blank (zero correction).
R_y = Colorimetric reading for diketogulonic acid.

No correction is necessary for ascorbic acid since no colour is obtained after an hour at 25° with quan-

tities less than 2 mg. and such high concentrations usually fall outside the range.

Table 1 gives the degree of accuracy that can be attained by determining diketogulonic acid in concentrations of about 0·5–5 mg. in 100 ml. solution.

Table 1. *Determination of diketogulonic acid in the presence and in the absence of dehydroascorbic and ascorbic acids*

Diketogulonic acid		Diketogulonic acid in the presence of dehydroascorbic and ascorbic acids			
		Dehydro-ascorbic acid	Ascorbic acid	Diketogulonic acid	
Added mg./100 ml.	Found mg./100 ml.	Added mg./100 ml.	Added mg./100 ml.	Added mg./100 ml.	Found mg./100 ml.
5·5	5·5	3·3	0	4·4	4·4
5·0	5·0	5·5	0	3·3	3·3
4·4	4·4	3·3	0	2·2	2·3
3·8	3·7	5·5	0	1·1	1·1
2·5	2·6	5·5	0	0·55	0·6
2·2	2·3	3·3	22·0	3·3	3·4
1·2	1·1	4·4	22·0	2·2	2·3
1·1	1·1	5·5	22·0	1·1	1·1
0·55	0·5	5·5	22·0	0·55	0·6

In this and in the following tables diketogulonic acid and dehydroascorbic acid are expressed as equivalent quantities in mg. of ascorbic acid from which they were prepared.

Effect of pH on the colour production. In studying the colour production in the presence of buffer solutions it was observed that the intensity of the colour produced by a given concentration of diketogulonic acid varied with the quantity of buffer added. This point is illustrated by the results given in Table 2. It was evident that the reason for the

Table 2. *Effect of buffering capacity on colour production*

Amount buffer, ml. in 9 ml. test solution containing 0·3 mg. diketogulonic acid	1	2	3	4	5
% Colour produced	102	108	113	114	122

Phosphate buffer *p*H 7·4; 2·72 g. KH_2PO_4 + 15·9 ml. *N* NaOH in 200 ml. solution

The colour intensity for 0·3 mg. diketogulonic acid obtained from the calibration curve = 100%.

deviation in the colour production was the *p*H of the reacting solution after the addition of the dinitrophenylhydrazine. This difficulty can be overcome by adjusting the *p*H of the test solution to about 2. Table 3 gives the results of an experiment in which diketogulonic acid (0·3 mg./9 ml.) in a number of buffered solutions of various *p*H's as well as in solutions containing different quantities of HCl, was determined by the above procedure before and after the adjustment of the test solution to *p*H 2. It is seen that the intensity of the colour produced increased with the rise in the *p*H of the test solution.

Table 3. *Influence of pH of test solution on colour production*

Buffer or acid added	Quantity ml. in 9 ml. test solution	*p*H	Colour production %	
			Before adjustment of *p*H	After adjustment to *p*H 2
Phosphate-citrate buffer*	2	4·0	105	100
" "	2	5·0	107	101
" "	2	6·0	109	101
" "	2	7·0	117	100
Borate buffer†	2	7·4	115	99
Phthalate buffer‡	2	6·0	107	101
N HCl	0·5	—	94	101
"	1·0	—	88	100
"	2·0	—	74	101

* McIlvaine [1921]. † Palitzsch [1915].
‡ Clark & Lubs [1916].

The colour intensity for 0·3 mg. diketogulonic acid obtained from the calibration curve = 100%

As the results of the preceding experiment (Table 2) show, this difficulty does not hold if the buffering capacity of the test solution is low, since the addition of the phenylhydrazine reagent then brings about a *p*H similar to that which obtains in the pure diketogulonic acid solution, which is used as the standard for comparison, after the addition of this reagent.

DETERMINATION OF DIKETOGULONIC ACID IN BIOLOGICAL MATERIAL

Blood and plasma. In view of the observations made in the preceding section it became evident that in preparing trichloroacetic acid extracts of tissues for the determination of diketogulonic acid, the quantity of the acid present in the extract would have to be considered. Fortunately it was found that up to 4 ml. of 5% trichloroacetic acid the intensity of the colour was not affected. An equal volume of 10% trichloroacetic acid was therefore added to guinea-pig whole blood or plasma into which a known quantity of diketogulonic acid had been previously introduced. After removing the precipitate by centrifugation, aliquot portions of the

Table 4. *Determination of diketogulonic acid in whole blood and plasma*

Whole blood mg./100 ml.		Plasma mg./100 ml.	
Added	Found	Added	Found
2·5	2·37	2·5	2·4
5·0	5·0	5·0	5·4
10·0	9·5	10·0	10·2
15·0	14·3	15·0	16·0
20·0	20·4	25·0	24·0
33·0	32·5	34·0	35·0
40·0	39·2	50·0	49·5
66·0	64·0	67·0	68·0
100	101	80·0	82·0
200	196	100	100

extract, not exceeding 4 ml. were submitted to the procedure described above. As the extracts of the whole blood or of the plasma gave a small but significant colour (equivalent to about 2 mg./100 ml.) with dinitrophenylhydrazine, it was necessary to determine the intensity of this colour in a control experiment, so that a correction could be made. Table 4 gives 10 representative determinations. The standard error of percentage deviation in concentrations from 2·5 to 200 mg./100 ml. of blood or plasma was found by us to be 3½%.

Liver, kidney and muscle. The tissues obtained from normal guinea-pigs, the added diketogulonic acid and 2 parts by weight of 10% trichloroacetic acid were ground with sand in a mortar, squeezed through muslin, reground, and centrifuged in the usual way. The sensitivity of the determination in this case was conditioned by the fact that the use of too much extract produced turbidity after the addition of the alkali. We nevertheless found that it was possible to determine 0·15 mg./g. of tissue with a standard error of percentage deviation of 4¼% after correcting for the blank. Results chosen at random are given in Table 5. The approximate equivalents of diketogulonic acid yielded by the blanks were, muscle 0·1 mg./g.; kidney 0·15 mg./g.; liver 0·2 mg./g.

Table 5. *Determination of diketogulonic acid in liver, muscle and kidney*

Liver mg./g.		Muscle mg./g.		Kidney mg./g.	
Added	Found	Added	Found	Added	Found
0·15	0·14	0·15	0·15	0·15	0·16
0·20	0·21	0·20	0·21	0·20	0·21
0·25	0·26	0·25	0·26	0·25	0·23
0·50	0·51	0·50	0·52	1·00	1·04
0·75	0·75	0·65	0·64	1·00	0·98
1·00	1·02	1·00	0·95	1·50	1·56
1·00	0·98	1·00	1·04	2·00	1·98
1·25	1·30	1·25	1·30	2·00	2·09
1·50	1·60	1·50	1·52	3·00	3·00
1·50	1·60	2·00	1·94	3·00	3·15

Urine. The diketogulonic acid was added to freshly voided human urine and the determination was carried out directly on the urine without previous extraction. When necessary a correction was made for the blank. This was never higher than the equivalent of 5 mg. of diketogulonic acid per 100 ml. of urine. Table 6 gives some results obtained with concentrations 2·5–40 mg./100 ml. of urine. The standard error of percentage deviation of all the urine determinations carried out by us was 3%.

Table 6. *Determination of diketogulonic acid in urine*

Added, mg./100 ml.	2·5	5·0	10·0	10·0	15·0
Found, mg./100 ml.	2·5	5·0	10·2	9·6	15·2
Added, mg./100 ml.	20·0	20·0	25·0	30·0	40·0
Found, mg./100 ml.	20·0	19·4	24·7	29·0	40·0

We should like to take this opportunity of expressing our gratitude to Dr R. G. Hatton, Director of East Malling Research Station, for laboratory accommodation. Thanks are also due to Messrs Roche Products Ltd. for a gift of ascorbic acid. One of us (J. R. P.) is indebted to the Medical Research Council for a whole time grant.

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Urea as a Partial Protein Substitute in the Feeding of Dairy Cattle

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The belief that certain simple nitrogenous compounds such as urea are capable of supplying to the ruminant a significant proportion of its nitrogen requirement is many years old. The theory most commonly advanced to account for the utilization of these simple compounds is that they are built up into protein by certain micro-organisms of the rumen which use them as a source of nitrogen for their own multiplication. The protein so formed is subsequently broken down in the small intestine to

amino-acids which are absorbed into the blood stream of the host along with those resulting from the digestion of the diet.

This theory has never been fully substantiated. Many feeding trials were carried out, mainly on the Continent, in the first three decades of the present century, but insuperable difficulties are encountered when an attempt is made to interpret the results of most of the experiments. In some instances the periods in which the non-protein nitrogen was fed were too short to enable reliable conclusions to be drawn. In others the periods were longer but the body-weights of the animals were not recorded, and again in others the controls were inadequate. These difficulties were emphasized by Krebs [1937] who, in reviewing the literature up to 1936, cited over 100 references and stressed the highly controversial nature of the results.

Since 1936 further publications have appeared suggesting that at least a portion of the dietary protein of ruminants can be replaced by substances such as urea. Hart, Bohstedt, Deobald & Wegner [1939] found that urea and ammonium bicarbonate, when added to a diet poor in protein and fed to growing calves, caused gains of live weight which were larger than those produced by the untreated diet, although the increases were slightly smaller than those obtained by the addition of the same amount of nitrogen in the form of casein. Bartlett & Cotton [1938] found no significant differences when one-third of the protein in the ration of young dairy heifers was replaced by urea, though they believed that the urea diet was slightly inferior to the protein diet. These publications, together with several others, have been discussed recently by Benesch [1941] and reviewed by Owen [1941]. It may be noted that they are concerned mainly with the effect of feeding on the growth of young stock or sheep, and that none of the work published in recent years has dealt with the value of these simple nitrogenous compounds for milk production.

The controversial nature of results and the war-time shortage of feeding-stuffs appeared to justify a thorough reinvestigation of the dietary value of urea for milk production. The general plan of attack and the objects in view have been outlined briefly by Owen, Smith & Wright [1941]. One section of the work was to consist of metabolism trials with lactating cows, in which urea would be compared with blood meal as a source of one-third of the nitrogen of the production ration. In the second section use was to be made of a steer with a large rumen fistula to determine by both in vivo and in vitro methods whether protein synthesis could actually be observed in the contents of the rumen and, if so, to study the factors affecting the magnitude and efficiency of such synthesis.

The present communication is limited to an account of the metabolism trials and their results.

OUTLINE OF THE EXPERIMENTS

Seven Ayrshire cows of average milk yield were used for the experiments. They were first fed a production ration in which a third of the nitrogen was supplied by blood meal, a product which has been shown by Morris & Wright [1933*a*] to contain a protein of first-class biological value for milk production. After the animals had been receiving this diet for a few weeks, the blood meal was replaced by its nitrogen equivalent of urea plus its energy equivalent as pure starch. With the first two cows the urea was fed for a period of 6 weeks and then replaced by blood meal. With the remaining five cows a period of about a fortnight without either urea or blood meal was inserted immediately after the first urea period. Each dietary change from one period to another was made gradually over 5 days. Further details as to the dates of the various periods and their duration are given in Table 1.

The animals were kept in special stalls in the metabolism house which has already been described by Morris & Wright [1933*b*]. The stalls were so fitted that by means of a continuously moving belt the urine and faeces of the cows could be collected separately with only slight mutual contamination. The faeces were weighed and sampled every 2 days, but the vessels for collecting urine were emptied each day and the urine sampled. Toluene was used to minimize bacterial action in these vessels. Composite 2-day samples of urine were then made for analysis. The slight contamination of the urine with faeces imparted to the urine a marked 'urease activity', with the result that part of the excreted urea was liable to be converted to ammonia in the collecting vessels. This rendered the estimation of urea itself relatively valueless and it was found necessary to estimate urea and ammonia nitrogen together. Preliminary tests showed, however, that this conversion of the urea did not result in any loss of nitrogen from the urine.

The milk yields were recorded daily, and with the first five cows composite 2-day samples were analysed for all the major constituents. With the remaining two cows the analysis was carried out at less frequent intervals. Urea and non-protein nitrogen were also determined at suitable times throughout the experiments in both the blood serum and the milk.

Two of the animals were weighed at the beginning and end of the experiment, and the remaining five at the beginning and end of each period.

The basis of rationing for maintenance was 5·5 lb. starch equivalent and 0·5 lb. protein equivalent per 1000 lb. live weight. For milk production the allowance was 2·5 lb. starch equivalent and 0·45 lb. protein equivalent per gal. The production ration per gal. of milk consisted of 0·25 lb. blood meal, 2 lb. oats, 1½ lb. bran, 0·25 lb. treacle and 0·5 lb. starch.

In Exp. 1 the maintenance ration for cows A and B consisted of 28 lb. swedes, 14 lb. straw and 2 lb. oats per day. It was found in this experiment that swedes were technically undesirable since they were not always completely consumed by the animals and since they contained a somewhat large proportion of non-protein nitrogen of which the nutritional value cannot at present be accurately assessed. In the experiment with cows C and G, a maintenance ration consisting of 13·5 lb. hay (6 % crude protein content) and 2·12 lb. potato starch per day was therefore fed, and the bran in the production ration was reduced by 0·5 lb. to compensate for the resulting slight increase of protein equivalent. In Exps. 1 and 3 a reduction was made in the production ration during the course of the work to parallel the decrease in milk yield. The point at which this change was made is shown in Fig. 1. In Exp. 2 the production ration was fed at the same level throughout.

The constituents of the production ration and the oats or starch from the maintenance ration were weighed each day and then thoroughly mixed in a mechanical mixer, the urea being dissolved in the molasses plus an equal volume of water. This procedure avoided any risk of localized concentrations of urea. The mixture was fed twice daily. There were no refusals of food during the urea periods, which indicates that the mixture was quite palatable. In Exp. 2 the hay was sampled and analysed at frequent intervals to ensure that the nitrogen intake was maintained at the correct level. The hay in Exp. 3 and the mixed diet in Exps. 2 and 3 were analysed every 2 days.

In all Kjeldahl determinations a mixed catalyst of potassium and copper sulphates and powdered selenium was used. Sulphur in the urine was determined by a modification of the method of Wolf & Osterberg [1910]. After having been boiled with conc. HNO_3 under a reflux condenser, the urine was subjected to a second oxidation with Denis's [1910] copper nitrate reagent. The total sulphur was then determined gravimetrically as $BaSO_4$. This method gave results agreeing well with those obtained by oxidation with a mixture of sodium carbonate and sodium peroxide. Urea and ammonia in the urine were determined by the urease method of Van Slyke and Cullen as described by Peters & Van Slyke [1932]. Before the determination of ammonia the samples of faeces were subjected to the action of urease.

RESULTS AND DISCUSSION

Milk yields

The milk yields for 2-day period for all seven cows are shown in Fig. 1, while the average yields in each period are recorded in Table 2. It will be seen from Fig. 1 that on feeding urea to cow A there was a decrease in yield from some 35 kg. to about 29 kg. during the first 16 days, a fall of 17 %. This decrease was, however, followed by a slight recovery, with

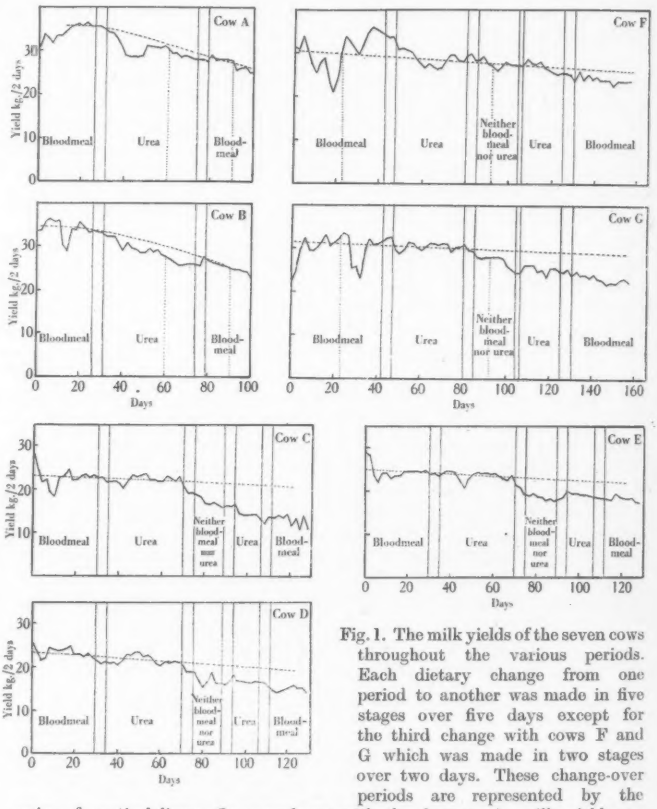

Fig. 1. The milk yields of the seven cows throughout the various periods. Each dietary change from one period to another was made in five stages over five days except for the third change with cows F and G which was made in two stages over two days. These change-over periods are represented by the pairs of vertical lines. In accordance with the decrease in milk yields, an appropriate reduction was made in the production ration fed to cows A, B, F and G at the points denoted by the vertical dotted line.

Table 1. *Plan of experiments*

Exp. no.	Period no.	Duration days	Description
1	1	26	Blood meal
Cows A and B	2	44	Urea in place of blood meal
6. xii. 39 to	3	28	Blood meal
19. iii. 40			
2	1	30	Blood meal
Cows C, D and E	2	36	Urea in place of blood meal
15. iv. 40 to	3	12	Neither blood meal nor urea
22. viii. 40	4	14	Urea replaced in the diet
	5	18	Blood meal
3	1	43	Blood meal
Cows F and G	2	29	Urea in place of blood meal
3. iii. 41 to	3	17	Neither blood meal nor urea
7. viii. 41	4	17	Urea replaced in the diet
	5	25	Blood meal

Table 2. *The average milk yields during the various periods (kg./2 days)*

	Cow						
Period	A	B	C	D	E	F	G
1	34·4	34·0	22·8	23·3	24·6	30·3	29·6
2	30·6	31·5	22·3	21·5	23·6	28·7	30·4
3	27·0	25·0	17·6	17·3	18·7	27·9	26·9
4	—	—	14·9	16·8	19·2	26·9	25·3
5	—	—	13·3	15·0	18·2	24·1	23·1

the result that during the last 3 weeks of urea feeding the yield approached closely to a value which might reasonably have been recorded as normal had blood meal been fed throughout. In fact the averages quoted for cow A in Table 2 suggest that urea did not result in any very serious decrease in yield, if the normal fall in lactation is taken into account. The urea feeding period in this experiment coincided with the exceptionally cold spell in the early part of 1940, and it appears that the temporary fall in yield may have been due to adverse weather conditions. With cow B no such initial decrease took place and the efficiency of urea for milk production appears to have approached very closely to that of blood meal.

The diagrams for cows C, D, E and G and the averages in Table 2 all show that milk production during the 4 or 5 weeks of urea feeding was practically as high as that obtained with blood meal, and that on removing urea from the diet a marked decrease in milk yield at once ensued. Thus with these four cows, yields of some 22, 21, 23 and 30 kg. at the end of period 2 were rapidly reduced to 17, 17, 19 and 27 kg. by the withdrawal of urea from the diet. These decreases amount to between 10 and 20%. With cow F the milk yield in period 1 was so variable that it is difficult to estimate the effect of urea with any certainty, but this animal appears to have behaved like cow A in that there was an initial decline in yield followed by a partial recovery. On withdrawing urea from the diet, however, the yield in this instance did not decline appreciably. It will be shown later that of the two cows F and G which were observed at the same time under identical conditions, the wastage of the ingested urea was much greater with cow F than with cow G. This fact may explain why urea did not seem to be as effective with cow F as it was with G.

It will be observed from Fig. 1 and Table 2 that for cows C, D, E and G the replacement of urea, and subsequently of blood meal, in the diet, after the period in which neither was fed, did not cause the milk yield to return to its expected (normal lactation) value. This is not an unusual experience, for if the milk yield is once allowed to decrease through inadequate feeding it is most difficult, particularly in the latter half of the lactation, to increase it again merely by an improvement in the diet.

So far as milk yields are concerned it may therefore be tentatively concluded that of the seven cows, five were able to utilize urea for milk production with reasonable efficiency, though the results for the sixth cow (F) were not clear-cut. With the remaining cow (A) there was an initial fall in yield at the outset of urea feeding, but this only proved to be temporary, and was probably due to the severe weather conditions. It has been suggested [Kay, 1942] that the feeding of urea in more than very limited quantities may have a toxic effect. It is clear that in the present experiments no such effect occurred. Indeed, with four of the seven cows the yield was practically maintained at its initial level in spite of advancing lactation.

Body weights

The body weights, which are recorded in Table 3, were obtained by weighing each animal three times on one day and averaging the values so obtained.

Table 3. *Body weights in kg.*

	Cows				
	C	D	E	F	G
At beginning of 1st blood meal period	464	473	488	483	478
At end of 1st blood meal period	450	425	450	446	428
Change	−14	−48	−38	−37	−50
At end of 1st urea period	450	455	478	445	413
Change during this urea period	None	+30	+28	−1	−15
At end of period when neither urea nor blood meal was fed	448	436	454	435	402
Change during this period	−2	−19	−24	−10	−11
At end of 2nd urea period	449	464	457	434	410
Change during this period	+1	+28	+3	−1	+8
At end of 2nd blood meal period	479	489	479	419	402
Change during this period	+30	+25	+22	−15	−8

Cows A and B were weighed only at the beginning and the end of Exp. 1. Their initial weights were 442 and 410 kg. respectively and their final weights were 394 and 409 kg.

The three weighings were made at 6 a.m., noon and 6 p.m. This precaution was taken to ensure that the average weight recorded for any one day was affected as little as possible by the ingestion of food and water or by the excretion of faeces and urine. Except on one occasion the differences between any of the three weighings on one day never exceeded 17 kg. and were usually much less.

It will be seen that the weights of all five cows in Exps. 2 and 3 decreased slightly during the first blood meal period, but that during period 2, when urea was substituted for blood meal, this tendency was checked, and in two instances (D and E) the decrease was converted to an increase. When, however, urea and blood meal were both withheld (period 3), the weights of cows D and E again showed a tendency to decrease in spite of the fact that the period was short and that it was accompanied by a reduction in milk yield. When urea feeding was resumed in period 4 these decreases in weight were again checked, and in period 5, except in the case of cows F and G, they were converted to gains.

It may be concluded that the urea feeding did not cause any significant loss of body weight, and that in certain instances gains were observed during the urea periods.

Nitrogen excretion

At 2-day intervals throughout the experiments composite samples of urine and faeces were analysed for total nitrogen and urea + ammonia nitrogen, and for creatinine and creatine. In the case of cows A and B urinary sulphur was also determined. Owing to the need for economy in space it is impossible to present here the large number of results obtained in this section of the work. Details of two typical examples are given in Fig. 2. Cow A gave similar results to cow B, while the results for cow C were representative of the remaining five animals. The average values for the intake and output of nitrogen and for the urea + ammonia excretion for Exps. 2 and 3 are shown in Table 4. It will be relevant to discuss at this point the metabolic data.

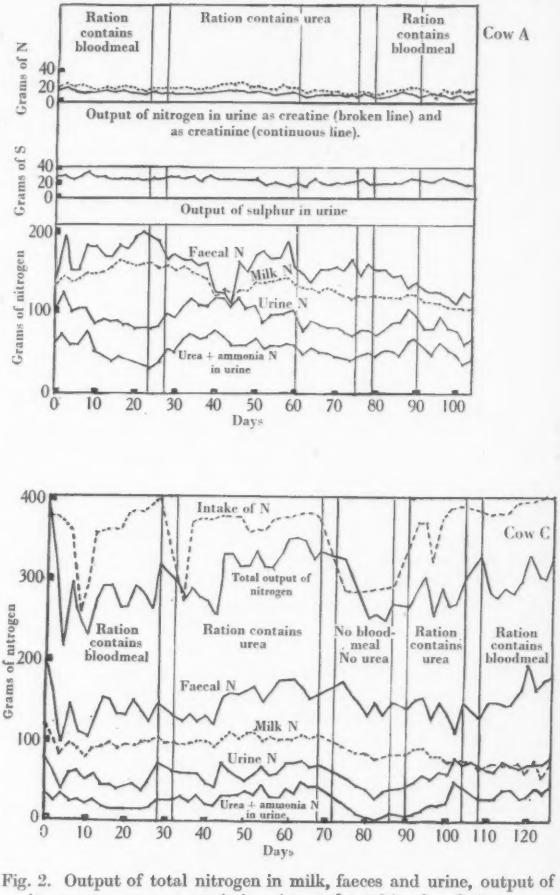

Fig. 2. Output of total nitrogen in milk, faeces and urine, output of nitrogen as urea + ammonia in urine, and total intake of nitrogen.

Nitrogen balances

It will be seen from Table 4 that, except in period 3, when both urea and blood meal were withheld, the animals appeared to be in a state of positive nitrogen balance to a somewhat remarkable degree. This was particularly true for cows C, D and E, the apparent positive balances attaining in some instances a figure of 90 g./2-day period. In view of the relatively small changes in the body weights of the animals (Table 3) it is difficult to regard these values as true indications of the precise state of nitrogen balance. We are unable to explain the tendency for our technique apparently to indicate such large positive balances. Each step of the procedure has been examined in detail with a view to solving this problem, but so far no solution has been found. It may be noted that in the work of Morris & Wright [1933*b*] and of Morris, Wright & Fowler [1936], in which the same technique was used, positive balances of as much as 50 and over 70 g. nitrogen per 2-day period were en-

countered in extreme instances, whereas negative balances occurred infrequently and were usually very small.

We are, however, of the opinion that the figures recorded for the balance in any one period can be compared in a relative sense with the corresponding figures for other periods. Thus with cow C the figures of +76·9, +53·2 and +11·4 for periods 1, 2 and 3 indicate that a probable positive nitrogen balance when blood meal was fed was slightly reduced when the blood meal was replaced by urea and was very much further reduced (if not rendered negative) when neither urea nor blood meal was ingested. It seems reasonable to apply a similar method of interpretation to all the nitrogen balance figures in Table 4.

It was again restored, however, when urea feeding was resumed.

The fact that the nitrogen balance was so much more positive in the two main urea periods than in the intervening nitrogen-deficient period indicated either that the urea-nitrogen was retained and anabolized by the animals or that the presence of urea in the diet in some way rendered the other nitrogenous constituents of the food more readily available for the animals' use. In this latter connexion a study of the values recorded in Table 4 for the faecal nitrogen of periods 1 and 2 does not suggest that the amount of nitrogen absorbed by the animal for metabolic purposes from the other constituents of the food was greater during urea feeding than it was with blood meal. In other words the apparent

Table 4. *Nitrogen excretion (g. nitrogen/2 day period)*

Period	Urine	Faeces	Milk	Total	Nitrogen intake	Nitrogen balance	Urea + NH_3-N in Urine	Urea + NH_3-N in Faeces	Urea + NH_3-N in Total	Creatinine g./2 days	Creatine g./2 days
Cow C											
1	52·0	136·1	94·9	283·0	359·9	+76·9	21·4	10·3	31·7	11·1	10·0
2	61·5	150·6	101·8	313·9	367·1	+53·2	32·9	15·6	48·5	10·3	5·8
3	42·7	147·6	84·1	274·4	285·8	+11·4	10·7	7·0	17·7	12·2	2·2
4	61·2	139·4	80·8	281·4	370·2	+88·8	25·3	10·5	35·8	13·3	5·8
5	68·8	161·8	67·4	298·0	390·4	+92·4	35·0	11·1	46·1	13·0	2·2
Cow D											
1	36·8	167·5	112·1	316·4	405·0	+88·6	18·3	17·9	36·2	6·2	7·8
2	71·1	172·5	109·1	352·7	404·0	+51·3	49·3	22·4	71·7	7·5	10·8
3	43·9	152·3	93·1	289·3	287·7	− 1·6	10·6	12·2	22·8	12·3	9·2
4	72·9	138·8	95·0	306·7	398·8	+92·1	45·9	12·2	58·7	9·0	12·5
5	76·1	164·9	88·0	329·1	418·8	+89·7	48·9	14·3	63·2	8·6	7·8
Cow E											
1	57·8	166·5	103·0	327·3	422·1	+94·8	30·4	19·5	49·9	11·7	8·7
2	90·3	166·8	108·3	365·4	406·6	+41·2	57·6	18·1	75·7	12·6	10·1
3	51·9	152·2	85·5	289·6	301·8	+12·2	11·4	9·0	20·4	16·1	7·3
4	102·2	132·2	92·8	327·2	424·0	+96·8	63·2	11·0	74·2	16·3	9·9
5	98·0	162·2	91·4	351·6	450·0	+98·4	60·8	12·8	73·6	12·7	5·4
Cow F											
1	96·0	162·0	120·9	378·9	388·1	+ 9·2	50·1	12·5	62·6	15·3	11·1
2	143·6	146·8	120·9	411·3	433·9	+22·6	95·8	15·5	111·3	14·9	15·9
3	59·4	127·2	115·0	301·6	298·2	− 3·4	24·8	6·9	31·7	14·5	10·3
4	88·1	133·6	117·8	339·5	356·5	+17·0	52·9	11·2	64·1	15·1	16·3
5	72·7	165·4	105·9	344·0	368·9	+24·9	34·8	9·4	44·2	14·3	11·3
Cow G											
1	67·4	174·0	131·9	373·3	398·3	+25·0	40·3	19·4	59·7	9·3	10·2
2	93·1	162·9	139·4	395·4	436·0	+40·6	57·1	21·9	79·0	9·1	12·8
3	44·2	139·6	123·7	307·5	302·9	− 4·6	15·7	9·8	25·5	8·5	10·6
4	71·3	138·3	122·8	332·4	365·0	+32·6	39·2	16·6	55·8	11·8	16·4
5	45·3	175·8	112·8	333·9	364·7	+30·8	16·9	14·3	31·2	9·6	8·6

If this method of interpretation is valid, two main conclusions may be drawn. (1) With three cows (C, D and E) the positive nature of the balance in the first urea period was significantly less than in the first blood meal period. In the case of the other two cows (F and G) the reverse was true. On the other hand, with all five animals the positive nature of the balance was very considerably reduced during period 3, when neither urea nor blood meal was fed.

utilization of urea cannot be ascribed solely to an increase in the digestibility of the other constituents of the urea ration.

Krebs [1937] has stated that an apparent retention of urea such as has been found here might merely be due to a swamping of the tissues with urea and not to a conversion of urea to protein. But if this were true one would expect urea to be equally effective in the diet of both the ruminant and the non-

ruminant animal, whereas it has been well established that in the latter ingested urea is of no nutritional value. Moreover, as a simple calculation will show, it is unreasonable to suppose that the large retention of urea-nitrogen observed in the present experiments could ever be explained by a swamping of the tissues with urea. If it be assumed that the amount of water in which urea can dissolve in the body of the cow is 75% of its body weight and that the concentration of urea in this water is the same as that in the blood serum, for which the maximum value found in this work never exceeded 15 mg. urea-N/100 ml. serum, then a 500 kg. cow would retain only about 120 g. urea. But in the whole of the second period of these experiments the total amount of urea fed to each cow was of the order of 3000 g. Even if the level of urea in the tissues could rise well above that in the blood, it is difficult to believe that it could ever attain a figure which would be sufficient to explain the observed findings, nor would such an hypothesis be capable of accounting for the maintenance of the milk yield and body weights.

Creatinine and creatine

Judging from recent publications by Bloch & Schoenheimer [1941], who find that urea is not a precursor of creatine, and by Beard, Espenan & Pizzolato [1939], who claim that it is a precursor, any variations which might be found in the amount of creatinine and creatine excreted in the urine of the ruminant, either on a blood meal or urea diet, would be difficult to interpret. It was, however, felt that a study of the creatinine-creatine excretion might give some useful information regarding the metabolism of the animals ingesting urea. The urinary excretion of creatinine and creatine was therefore estimated for all seven animals by the usual colorimetric procedure of Folin. For the first two cows the coloured solutions were compared with the standard in a visual colorimeter. For the others a 'Spekker' photo-electric absorptiometer was used. The results for cow A, which were very similar to those for cow B, are shown in Fig. 2. The average values for cows C to G are recorded in Table 4. It is impossible to draw any definite conclusion from the results, for the variations from one 2-day period to another were usually too large to enable inter-period variations to be detected. Nor was there any appreciable change when both urea and blood meal were withheld. It seems probable that really substantial inter-period changes in these values can only be observed with cows subjected to relatively severe inanition such as that described by Morris & Ray [1939]. Further work on this subject is, however, clearly desirable, since the results do not fall into line with those published earlier by Morris & Wright [1933*a*, *b*].

Diuresis

Since urea is a diuretic agent it is important to determine whether urea-feeding with ruminants is liable to cause excessive and harmful diuresis. The average weight of urine excreted per 2-days in the various periods is shown for all seven cows in Table 5.

Table 5. *Average weight of urine excreted in the various periods (kg./2 days)*

	Cows						
Period	A	B	C	D	E	F	G
1	15·36	9·63	6·28	5·25	5·25	12·89	8·62
2	12·99	10·55	7·11	11·96	9·62	12·65	9·68
3	12·75	8·32	7·31	9·01	7·54	8·73	7·55
4	—	—	9·05	12·51	14·35	7·83	8·22
5	—	—	8·88	10·28	9·34	7·80	6·72

With cows A and F there was no diuresis when urea replaced blood meal in the diet. With the other cows, however, in which the weight of urine was initially much less, increased excretion did occur during the urea periods. This was most marked with cow E, in which a value of 5·25 kg. in period 1 rose to 9·62 kg. in period 2. Again, the value of 7·54 kg. in period 3, when both blood meal and urea were withheld, increased to 14·35 kg. in period 4, when urea feeding was resumed. On the other hand, it should be noted that the figure of 9·62 kg. for the first urea period did not differ materially from that of 9·34 kg. for the final blood meal period, nor does the highest value of 14·35 kg. represent an abnormal excretion for a cow in 2 days. Smaller amounts were excreted in period 1 by cows A and F and again in period 5 by cow A. It therefore seems unlikely that urea, if used in a prepared production ration in which it accounted for some 30% of the nitrogen, would induce any harmful diuresis.

The non-utilization of a portion of the ingested urea

The results recorded in Table 4 show that the total amount of nitrogen excreted in the urine each 2 days during the urea periods was greater than that excreted during the blood meal periods, and that this excess could be accounted for mainly by an increase in the excretion of urea + ammonia-nitrogen. With cow D, for example, a value of 36·8 g. for the total urinary nitrogen during the blood meal period increased to 71·1 g. during the urea period, an increase of 34·3 g., the corresponding values for urea + ammonia-nitrogen in the urine being 18·3 and 49·3, a difference of 31·0 g. If, however, it were assumed that urea was quantitatively converted to protein in the rumen and that this protein became available to the animal to the same degree as blood meal, the excretion of urinary nitrogen would be expected to remain the same in the urea periods as it was in the blood meal periods. It seems probable therefore that

Table 6. *The proportion of ingested urea which does not appear to be utilized by the animal compared with the level of urea in the blood*

Cow	Urea-N ingested in period g./2 days		The amount by which the urinary N in period 2 exceeded that in period 1		The amount by which the urinary N in period 4 exceeded that in period 5		% of ingested urea which appeared to be wasted in period		Average value for urea-N in the blood serum mg. N/100 ml. during period		% increase
	2	4	Total urinary N g./2 days	Urea + NH_3-N g./2 days	Total urinary N g./2 days	Urea + NH_3-N g./2 days	2	4	1	2	
A	85·7	—*	13·0	13·7	—	—	15–16	—	4·5	8·3	84
B	91·0	—	23·0	14·1	—	—	15–25	—	6·3	10·5	67
C	85·9	93·7	9·5	11·5	− 7·6	− 9·7	11–13	None	4·7	5·8	23
D	93·7	93·8	34·3	31·0	− 3·2	− 3·0	33–36	None	4·5	7·3	62
E	99·6	99·7	32·5	27·2	+ 4·2	+ 2·4	27–33	2–4	6·2	9·0	45
F	99·4	81·6	47·6	45·7	+15·4	+18·1	46–48	19–22	—†	—	—
G	99·8	83·6	25·7	16·8	+26·0	+22·3	17–26	27–31	—	—	—

* There were only three periods with cows A and B.
† No blood analysis was carried out for cows F and G.

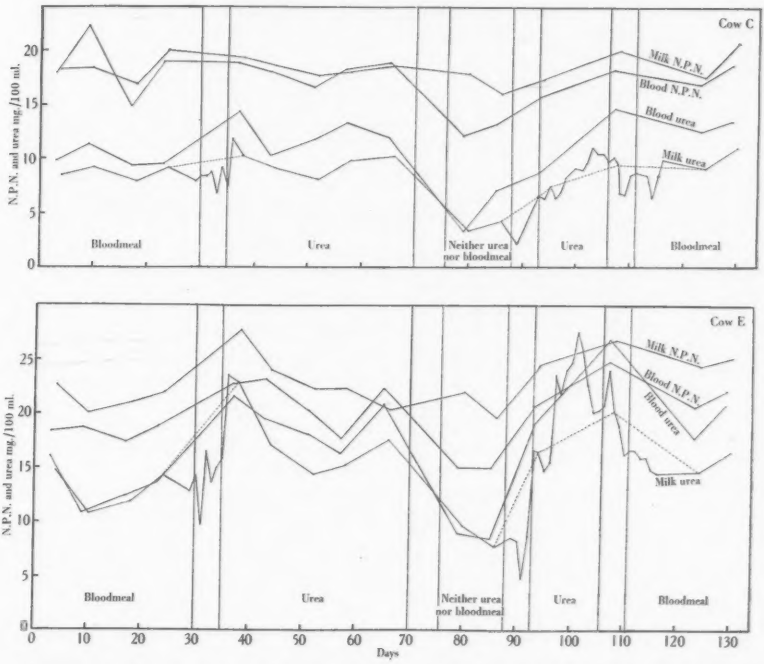

Fig. 3. The concentration of N.P.N. and urea in the blood and milk. For the five animals for which these data are available, cow C showed the least and cow E the greatest variation on replacing blood meal with urea. For this reason the results for the two cows have been chosen for illustration. The broken lines bring out the general parallelism between the concentration in the blood and that in the milk.

this increase in the nitrogen excretion when urea is fed may be taken as a measure of the amount of ingested urea which passes through the animal without acting as a protein substitute. In Table 6 the approximate amount of this unused urea is calculated. It should be noted that the value obtained for any one cow is roughly the same whether the total urinary nitrogen or only that excreted as urea + ammonia is used as the basis of calculation.

If the above reasoning is sound it may be concluded that the proportion of urea utilized varies greatly with different animals. With the seven cows

in the present work the proportion of urea passing through the animal unused in period 2 varied from 12 to 47 % and averaged 25 %. Moreover, it is important to observe that cow C, which showed the smallest wastage of urea, also showed the greatest benefit from the feeding of this substance, for the milk yield of this animal was maintained at as high a level in period 2 as in period 1, though removal of both blood meal and urea resulted in an abrupt decline. Again, cow F, which showed the least benefit in milk yield from urea feeding, also showed the greatest degree of wastage.

One further point should be noted. For cows C, D and E the values calculated for the wastage of urea in period 2, which followed a blood meal period, was very much higher than those calculated for period 4, in which urea feeding followed immediately after a period of 2 weeks during which both blood meal and urea were withheld. In fact, with cows C and D the nitrogen of urea appeared to be better utilized during period 4 than was that of blood meal fed in equivalent amounts during period 5. It appears from this fact that the proportion of the dietary urea which is wasted may depend to some extent on the nutritional state of the animal prior to urea feeding, a phenomenon which is not uncommonly encountered in other types of nutritional study. The same feature was observed with cow F, though not with cow G. It should be pointed out that these wastage values have been calculated on the assumption that the nitrogen of blood meal itself is completely utilized; they are therefore relative and not absolute.

Urea in the blood and milk

At intervals during the experiments urea and non-protein nitrogen were determined in the blood serum and milk of cows A to E and also in the milk of cows F and G. Typical results (those for cows C and E) are shown in detail in Fig. 3. It will be seen that the changes brought about by alterations in the diet were much smaller with cow C than with cow E. The average values of the blood urea for cows A to E during the first two periods are recorded in Table 6. During the urea period these exceeded those found during the blood meal period by amounts varying from 23 to 84 %. When both urea and blood meal were withheld from the diet a marked decrease in blood urea occurred (Fig. 3), and this was again followed by a substantial increase when urea feeding was resumed.

In all instances in which analyses of this type were obtained, there was a general parallelism between the concentration of N.P.N. in the blood and that in the milk, while the concentration of urea showed a similar relationship. It may be noted that the blood samples were taken within a period of minutes whereas the milk samples were representative of a complete 24 hr. secretion. The results confirm the earlier finding of Peskett [1934] that the urea content of cows' milk is almost identical with that of the blood.

In addition to these analyses, which were carried out at 5–10-day intervals, urea was also estimated on composite daily samples of the milk just before, during and after the change-over from period 1 to period 2, and also throughout period 4. Typical results are shown in Fig. 3. When urea was included in the diet immediately after blood meal feeding, the urea in the milk reached a maximum after 2–3 days. On the other hand, after period 3, when the diet was deficient in nitrogen, the time required for the same maximum to be attained was 8–10 days after the resumption of urea feeding.

Of the five cows for which blood urea values are available, the one which showed the least increase in the level of urea in period 2 (cow C) also showed the

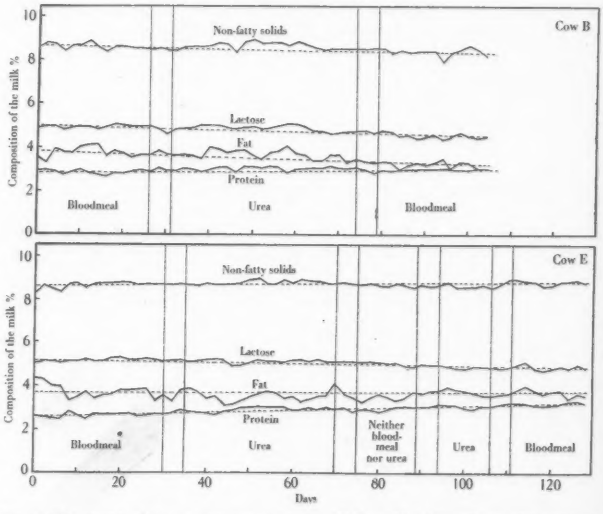

Fig. 4. The level of the major constituents of the milk throughout the various periods. The results for cow A were very similar to those obtained for cow B. The results for cow E are typical of those obtained for all the other five animals. In each instance the average values for the first and last periods have been plotted at points mid-way through these periods. The broken lines join these points.

least apparent wastage, and therefore the greatest utilization of this substance (Table 6). As regards milk yield (Fig. 1 and Table 2), this cow also benefited most from the inclusion of urea in its diet and suffered most from its withdrawal. For the other animals no definite correlation could be detected between blood urea levels and the apparent wastage of urea.

The composition of the milk

To both the producer and the consumer of milk it is important that data should be available to show whether the composition of the milk is adversely affected by the ingestion of urea. In the present experiments the highest concentration of urea in the milk was roughly 28 mg./100 ml., or 0·16 g. per pint (cow D, period 4); with the remaining cows it was very considerably below this level throughout most of period 2. Such an amount of urea is without effect on the taste of the milk and could not have any adverse effect on the consumer.

The principal constituents in composite 2-day samples of the milk were also determined for the first five cows, while for the remaining two similar determinations were made at less frequent intervals throughout the experiment. Two examples, which are typical of all the results, are shown in Fig. 4. They demonstrate clearly that urea-feeding did not bring about any alteration in the general composition of the milk.

SUMMARY

1. The value of urea as a partial substitute for dairy cows has been investigated by feeding it to seven lactating animals in an amount equivalent to 33% of the nitrogen in the production ration or to 25% of the total nitrogen intake. Data for milk yield, body weight, nitrogen balance and milk composition obtained while feeding this diet have been compared with the corresponding data obtained when the animals were receiving (*a*) blood meal and no urea and (*b*) neither blood meal nor urea.

2. The milk yields of five of the seven animals were well maintained when blood meal was replaced by urea. With four of the five cows under test a rapid and significant decrease in milk yield took place when urea was removed from the food. For one of the remaining two cows the graph of the milk yield was difficult to interpret owing to an initial decrease during the first few days of urea feeding. This decrease may have been due in part to the exceptional weather conditions then prevailing. The seventh cow did not appear to benefit to any significant extent from the inclusion of urea in its food.

3. Changes in body weights were not marked at any stage of the experiment, but on the whole they seemed to be better maintained on urea than on blood meal.

4. The nitrogen balance and excretion data show that, although urea was partially retained by all the animals, its retention was not complete. Compared with blood meal, amounts varying from some 12 to 47% and averaging 25% of the ingested urea apparently passed through the animal without being utilized. This apparent wastage was much reduced when urea feeding was preceded by a period in which the diet had been deficient in total nitrogen. The quantitative importance of this failure to utilize all the dietary urea is discussed.

5. The urea content of the blood tended to increase at the outset of the urea period and then to return towards its initial value as the urea feeding progressed. The urea content of the milk closely approximated that of the blood and never exceeded 28 mg./100 ml. (0·16 g. per pint), an amount which would not have any deleterious effect on the consumer.

One cow showed very little change in the urea content of its blood and milk at any time during the experiment. This animal also appeared to utilize the ingested urea to the greatest degree and to benefit most from its inclusion in the diet.

6. The ingestion of urea had no measurable effect on the percentage of protein, fat, lactose or total solids in the milk.

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The Application of the Spekker Photoelectric Absorptiometer to the Determination of Vitamin C

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In a footnote to their recent paper on the reliability of the chemical estimation of vitamin C by titration with 2:6-dichlorophenolindophenol, Harris & Olliver [1942] mention the application of a procedure involving the addition of an excess of dye and instantaneous measurement of the excess of colour, in order to differentiate the rapid reduction effected by ascorbic acid from the slower reduction due to non-specific reductants. Several methods employing this principle have already been described [Meunier, 1937; Guthe & Nygaard, 1938; Evelyn, Malloy & Rosen, 1938; Bessey, 1938; Bukatsch, 1939]; in every case, however, the authors have relied on measurements made on a single mixture of test solution and dye. The measurement is complicated by the fact that, in the presence of non-specific reductants, the colour is fugitive owing to the progressive decolorization of the excess dye by the interfering materials. Meunier [1937] investigated kinetically the decolorization of 2:6-dichlorophenol-indophenol by ascorbic acid, glutathione and cysteine and concluded that in the presence of the latter substances the loss of colour due to ascorbic acid could be satisfactorily assessed by making a plot of residual colour against time and extrapolating back to zero time. Subsequently other workers have also employed this device to allow for the fugitive character of the excess dye coloration.

When a Spekker photoelectric absorptiometer was employed for the determination of ascorbic acid in fruits and jams by this method it was found that fading of the colour of excess indophenol was sometimes very rapid, especially with solutions which had been treated with hydrogen sulphide for the estimation of the partially oxidized dehydroascorbic acid [McHenry & Graham, 1935]. Accuracy of extrapolation in such cases was not high and for this reason it was considered inadvisable to rely on readings of the colour due to a single addition of indophenol. An improved method, involving readings on a number of solutions with different amounts of excess indophenol, has therefore been developed.

METHOD

To a series of increasing amounts of a solution of 2:6-dichlorophenolindophenol, each diluted with distilled water to a fixed volume, are added uniform aliquot portions of the test solution. The colour of each mixture is measured by means of a Spekker photoelectric absorptiometer, a further similar aliquot of the test solution diluted to the same volume with distilled water only being used as a standard. The reading on each mixture is corrected for fading by making three observations over about a minute and extrapolating back to zero time. The colour values of the mixtures after correction are then plotted against the amount of indophenol reagent added; the appearance of excess reagent is marked by a sudden increase in the slope of the resulting curve, the point of flexure corresponding with the end-point of the titration.

Solutions employed. (*a*) *Indophenol reagent.* A solution of 2:6-dichlorophenolindophenol containing 0·25 mg./ml. was found to be most suitable for the Spekker titration. To stabilize the dye 10% of Clark's phosphate buffer of *p*H 7·0 was incorporated. This reagent was stored in a dark bottle in the refrigerator; it was renewed after about a month and standardized every 2 or 3 days in the usual manner [Harris & Olliver, 1942].

(*b*) *Sample solution.* The sample solution, which may be prepared in any suitable manner, should contain preferably from 5 to 8 mg. and in any case not more than 10 mg. ascorbic acid per 100 ml.

Titration procedure. The Spekker instrument measures the residual light transmission of the test solution and in examining a series of solutions of progressively increasing colour the instrument reading diminishes continually. The instrument must therefore be adjusted to allow of a suitable range of readings being obtained before complete extinction is reached. To this end 4 ml. of test solution are added from a pipette with a rapid delivery to 4 ml. of distilled water in a 1 cm. Spekker cell and mixed in with a quick shake. The instrument is adjusted to give a reading of 1·000 on this mixture with the Spekker no. 6 light blue filter in position. If the sample solution is itself of a red colour, it may be found that the sensitivity of the instrument with the light blue filter is very low. The sensitivity can be improved by substituting a green or even a red filter, but the titration curve obtained is of much less satisfactory shape. It is preferable to retain the blue filter and balance the instrument as accurately as the reduced sensitivity will permit.

The mixture in the cell thus prepared is retained as a 'zero' check.

Readings are then made on a series of solutions to which progressively increasing amounts of indophenol reagent have been added, the amount of water being correspondingly reduced to keep the total volume at 8 ml. in each case. The end-point of the titration must be known approximately, either from a preliminary titration or from previous experience with the particular material under examination, and the additions of indophenol reagent should be chosen to allow at least two additions before the end-point is reached. After the end-point, at least five further additions should be made, the amount of indophenol reagent being increased by 0·2 ml. each time. The observations on these solutions are made in the following manner.

To the appropriate indophenol reagent-water mixture in a second 1 cm. Spekker cell are added 4 ml. of test solution from the pipette previously used and at the same time a stop-watch is started. The solutions are mixed with a quick shake and the instrument reading is observed as rapidly as possble; the reading and time are noted. The instrument is thrown out of adjustment and readjusted, and a second reading and time, and finally a third, are obtained in the same way. These readings should be made as rapidly as possible, especially if appreciable fading, indicated by a steady increase in the instrument reading, is observed; all three readings should be completed within a minute at the most. In difficult cases a convenient device to enable the first reading to be obtained rapidly is to set the instrument to the approximate reading expected, as judged from the previous readings, before adding the test solution to the indophenol reagent-water mixture. The 'zero' reading should be checked after the observations on each mixture.

Interpretation of results. A plot is first made of Spekker reading against time for each mixture examined and the curves so obtained are extrapolated back to zero time to indicate the probable initial Spekker reading of each mixture. A second plot is then made of the extrapolated initial Spekker readings against the amount of indophenol solution added for the whole series of mixtures examined. This second plot represents the variation in colour of the sample solution, diluted to a fixed volume, as progressively increasing amounts of 2:6-dichlorophenolindophenol are incorporated. It comprises two relatively straight portions: one, horizontal, corresponds to the more or less uniform colour of the mixtures in which all the colour of the added indophenol has been discharged by the ascorbic acid present; the other, inclined fairly steeply, corresponds to the progressively increasing colour of the excess indophenol. These straight portions intersect at a relatively sharp angle and the point of intersection corresponds with the end-point of the titration.

The accuracy of the determination of the point of intersection depends primarily upon the correct drawing of the steeper portion of the plot. In practice the individual results deviate somewhat from linearity owing to random fluctuations; it was thought possible, therefore, that visual assessment of the optimum straight line to represent these results might not be sufficiently exact to avoid the introduction of appreciable errors. This point was examined by comparing, for a number of typical plots, the results obtained by such visual assessment and by application of the method of least squares to the calculation of the slope of the steeper portion of the plot. Of seventeen results examined, only two showed a deviation exceeding 2%, while the mean deviation was $\pm 1\%$. Thus direct visual assessment, if carefully carried out, is sufficiently accurate for all practical purposes.

According to Meunier [1937] extrapolation to zero time is itself sufficient to eliminate errors due to interference by more slowly reacting non-specific reductants. If this were so, it would be possible, after a suitable calibration of the instrument, to calculate the ascorbic acid content of any mixture from its initial Spekker reading, obtained by extrapolation of experimental readings to zero time; methods of this type have, in fact, already been described [Evelyn *et al.* 1938; Bessey, 1938]. In practice, however, fading of the mixtures is sometimes extremely rapid, and this necessarily means that the accuracy of the extrapolation is correspondingly limited. In fact, as remarked above, the individual results on the steeper portion of the plot frequently deviate somewhat from the theoretical straight line, indicating that these individual results are not free from appreciable errors. The determination of the first appearance of excess indophenol, as indicated by the point of intersection on the plot, is subject to much less uncertainty. In this connexion it may be remarked that during the examination of a large number of materials by the method it was observed that, even when fading of the mixtures was very rapid and extrapolation correspondingly difficult, the shape of the final plot was always quite satisfactory.

EXAMINATION OF THE METHOD

Titration of cysteine and glutathione. Of the various non-specific reductants which may give rise to interference in the titration of ascorbic acid with 2:6-dichlorophenolindophenol, the most common are probably cysteine and glutathione. An examination of the effects of these materials on the estimation of ascorbic acid by the Spekker titration method was therefore made. In the first place titrations were carried out on solutions containing cysteine (in the

Table 1. *Titration of solutions of cysteine (hydrochloride) and glutathione with 2:6-dichlorophenolindophenol*

Amount present* mg./100 ml.	Indophenol required for 4 ml. test solution (ml.)			
	Cysteine (hydrochloride)		Glutathione	
	Visual titration	Spekker photoelectric titration	Visual titration	Spekker photoelectric titration
6·5	0·045 (1 drop)	Nil	0·045	−0·01
26·0	Pink colour after 1 drop, but fugitive in about 20 sec. Similar effect with further additions. Gradual increase in time required to discharge colour, but no true end-point	−0·04	0·045	Nil
65·0	Dye added in 0·2 ml. portions. Pink colour after first addition, but fugitive within 15 sec. Similar behaviour with subsequent additions, complicated by appearance of greenish yellow end-colour. Permanent pink colour at about 12 ml.	−0·04	0·045	Nil

* These figures represent the amount of cysteine present; the amount of hydrochloride taken was appropriately greater.

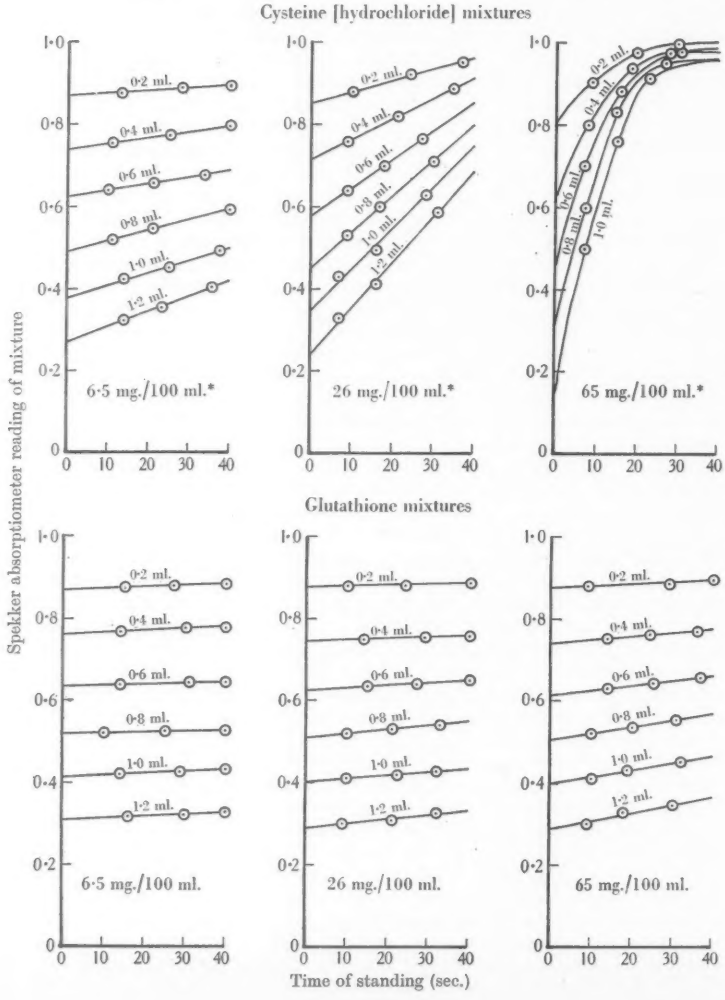

Fig. 1. Fading of solutions containing 2:6-dichlorophenolindophenol in admixture with cysteine (hydrochloride) and glutathione. (Figures attached to curves indicate amount of indophenol solution added to 4 ml. portion of cysteine or glutathione solution.) See note attached to Table 1.

form of its hydrochloride) and glutathione alone. The solutions were prepared in 6% acetic acid and contained approximately one, five and ten times the amount of ascorbic acid normally aimed at in the test solution; comparative visual titrations with indophenol were made. The results are given in Table 1.

Fig. 1 shows the fading curves of these mixtures; it is to be noted that, since the Spekker absorptiometer measures the amount of residual light trans-

Table 2. *Recovery of ascorbic acid in admixture with cysteine (hydrochloride) and glutathione*

(Paired results for the visual titration were obtained one before and one after the photoelectric titration)

Amount of interfering material present mg./100 ml.*	Ascorbic acid found, mg./100 ml. In presence of cysteine (hydrochloride)		In presence of glutathione	
	Visual titration	Spekker photoelectric titration	Visual titration	Spekker photoelectric titration
Nil	6·28, 6·35 } 6·32	6·10	6·28, 6·35 } 6·32	6·10
6·5	6·40, 6·40 } 6·40	6·16	—	—
26·0	6·52, 6·45 } 6·49	6·10	6·53, 6·40 } 6·47	6·13
65·0	7·8, 7·8 } 7·8†	6·10	6·40, 6·43 } 6·42	6·10

* See note attached to Table 1.

† This figure is very approximate: no definite end-point could be obtained.

It will be observed that even in the absence of any interfering material the visual titration yields higher results than the Spekker photoelectric titration; this is to be expected, since in a visual titration a small excess of indophenol is required to produce a visible colour, whereas the photoelectric procedure should yield the exact end-point of the titration. As the amount of interfering material increases, with its attendant difficulty in the observation of the visual end-point, the apparent recovery of ascorbic acid by the visual titration increases progressively. Recovery by the photoelectric method, apart from slight random variations, remains constant.

The photoelectric titration curves of the mixtures are given in Fig. 2: all the curves are of quite satisfactory shape, although the mixtures containing large amounts of cysteine showed very rapid fading (cf. Fig. 1).

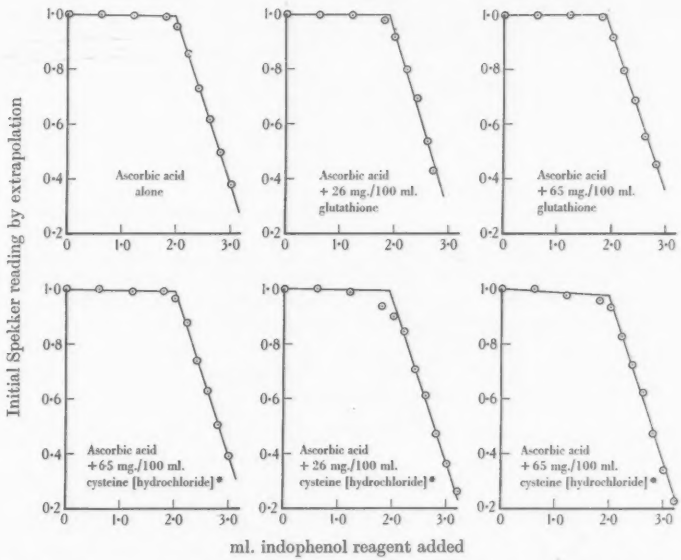
Initial Spekker reading by extrapolation
Ascorbic acid alone
Ascorbic acid + 26 mg./100 ml. glutathione
Ascorbic acid + 65 mg./100 ml. glutathione
Ascorbic acid +65 mg./100 ml. cysteine [hydrochloride]*
Ascorbic acid + 26 mg./100 ml. cysteine [hydrochloride]*
Ascorbic acid + 65 mg./100 ml. cysteine [hydrochloride]*
ml. indophenol reagent added

Fig. 2. Photoelectric titration curves of ascorbic acid in admixture with cysteine (hydrochloride) and glutathione. See note attached to Table 1.

mitted, a diminution of colour is indicated by an increase in the instrument reading. In both cases the rapidity of fading depends upon the concentration of each of the reactants; as indicated by the visual observations, however, fading of mixtures containing cysteine is very much more marked than that of mixtures containing corresponding amounts of glutathione; even in greatly excessive concentrations, fading due to the latter material is relatively slow. It is satisfactory that, even with the very rapid fading observed in the presence of large amounts of cysteine, the final plot approached closely to linearity and indicated only a slight negative departure from the theoretical zero titration value.

Recovery of ascorbic acid from mixtures containing cysteine and glutathione. Solutions were prepared in 6% acetic acid containing 6·5 mg./100 ml. of commercial synthetic ascorbic acid in admixture with varying amounts of cysteine (hydrochloride) and glutathione; a control solution was prepared containing ascorbic acid alone. These solutions were titrated with 2:6-dichlorophenolindophenol, both visually and by the Spekker photoelectric method. The results are given in Table 2.

APPLICATION OF THE METHOD TO NATURAL MATERIALS

The method has been applied to the examination of a range of plant materials, including both natural and manufactured products. Some of the materials examined apparently contained appreciable amounts of reducing substance other than ascorbic acid (as shown by the shape of the fading curves in the Spekker titration) and in these cases the Spekker titration gave considerably lower results than a visual titration with indophenol. Table 3 shows some typical results.

The lemon juice showed little variation between the results obtained by the two methods, the small difference observed being attributable to the excess of indophenol required to produce a visible colour. The other materials, however, showed considerable discrepancies and, since there seems no reason to suppose that the Spekker titration could yield low results, it appears that with these materials the visual titration may be considerably in error.

Table 3. *Comparison of visual and Spekker titrations of plant materials with 2:6-dichlorophenolindophenol*

Material	Ascorbic acid found, mg./100 g.	
	Visual titration	Spekker titration
Lemon juice	58·2	55·7
Unripe walnuts, solution in 5% metaphosphoric acid	1628.0	1415·0
Banana honey (concentrated banana juice), solution in 6% acetic acid	Fading prevented definite end-point. First colour corresponded to about 58 mg.; fairly permanent colour corresponded to 70–75 mg.	16·0

SUMMARY

The Spekker photoelectric absorptiometer has been applied to the determination of ascorbic acid in the presence of non-specific reductants by a step-titration method; satisfactory recovery of ascorbic acid was obtained in the presence of cysteine and glutathione in amounts up to ten times that of the ascorbic acid itself.

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Studies on Cholinesterase

1. CHOLINESTERASE AND PSEUDO-CHOLINESTERASE

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The existence of an enzyme capable of hydrolysing acetylcholine into choline and acetic acid, first suggested by Dale [1914], was established by Loewi & Navratil [1926] and has since been confirmed by many investigators.

It is still a matter of controversy whether the hydrolysis of acetylcholine is effected by a non-specific esterase or by a specific enzyme, which acts exclusively on esters of choline. Stedman & Stedman [1935], regarding the enzyme present in blood serum as the specific catalyst for the hydrolysis of choline esters, named it choline esterase. While not insisting on the absolute substrate specificity of the enzyme, Easson & Stedman [1937] maintain that cholinesterase is 'specific to the extent that its principal action is to hydrolyse simple esters of choline'. Vahlquist [1935], on the contrary, denies the specificity of cholinesterase in plasma, claiming that the hydrolysis of acetylcholine is brought about by the same enzyme which hydrolyses tributyrin. The specific nature of the enzyme in tissues, on the other hand, has, apparently, not been questioned, the hydrolytic action of tissue extracts on acetylcholine being indiscriminately referred to as 'cholinesterase activity'.

It is the purpose of this paper to show that there exist in the animal body two esterases capable of hydrolysing acetylcholine: a true cholinesterase acting exclusively on choline esters, and a non-specific enzyme, which hydrolyses not only esters of choline but a variety of non-choline esters as well. Moreover, experiments with both enzymes at high and low concentrations of acetylcholine, have revealed a decisive difference between the two esterases, calling for a sharp distinction of the true cholinesterase from the non-specific enzyme, for which we venture to suggest the name pseudo-cholinesterase.

1. PSEUDO-CHOLINESTERASE

The following three series of experiments, carried out with extensively purified enzyme preparations, are neither consistent with the conception of the specific nature of the 'cholinesterase' present in blood serum, nor do they support the view that the hydrolysis of choline esters by tissues is invariably due to a specific enzyme.

(*a*) *The action of 'cholinesterase' from serum and pancreas on non-choline esters**

Horse serum and crude tissue extracts from pancreas (dog) hydrolyse not only esters of choline, but also have a marked hydrolytic effect on non-choline esters such as tributyrin, tripropionin and methyl butyrate. Moderate purification of the 'cholinesterase' from these sources results in a considerable loss of activity towards non-choline esters; but once a hundred-fold increase in 'cholinesterase' activity has been attained, the ratio $Q_{Ach}/Q_{non\text{-}ChE}$ remains constant upon further purification. Even when the activity of the 'cholinesterase' has been increased about two thousand times this ratio is still the same (Table 1).

A variety of steps is required in order to proceed from a hundred-fold to a two thousand-fold increase in 'cholinesterase' activity. If we assume that two distinct enzymes are present in our hundred-fold purified extracts, the one acting on acetylcholine, the other on non-choline esters, it seems reasonable to expect that the ratio $Q_{Ach}/Q_{non\text{-}ChE}$ would change during the process of further purification. The fact that this ratio remains constant suggests that a single enzyme is responsible for the observed choline and non-choline ester hydrolysis. The following experiments verify this hypothesis.

* The method for the purification of the tissue 'cholinesterase' (dog pancreas) used in our experiments will be found in this issue [Mendel & Mundell, 1943]; the purification of the pseudo-cholinesterase from horse serum will be published later.

The activity of the enzyme preparation was measured manometrically by Warburg's method at 37·5° in 0·025 *M* $NaHCO_3$ saturated with 5% CO_2 (*p*H 7·4). Each molecule of acid liberated from choline esters or non-choline esters during hydrolysis, releases one molecule of CO_2 from the bicarbonate. Therefore, the quotient

$$\frac{[\text{volume of } CO_2 \text{ (in } \mu\text{l.)}]}{[\text{time (in hr.)}] \times [\text{dry-wt. of enzyme preparation (in mg.)}]},$$

which will be denoted by the symbol Q, is a measure of the activity of the enzyme preparation. The suffix to Q refers to the substrate: Ach = acetylcholine, Tb = tributyrin, Mb = methyl butyrate, Tp = tripropionin and non-ChE = non-choline esters.

Table 1. *Hydrolysis of choline and non-choline esters by 'cholinesterase' preparations of varying purity*

Substrate concentration 0·03 *M*

Source of 'cholinesterase'	Increase in 'cholinesterase' activity	Q_{Ach}*	Q_{Tb}†	Q_{Mb}	Q_{Tp}	$\frac{Q_{Ach}}{Q_{Tb}}$	$\frac{Q_{Ach}}{Q_{Mb}}$	$\frac{Q_{Ach}}{Q_{Tp}}$
Horse serum	0	34	60	30	—	0·57	1·13	—
,,	118-fold	4,000	1,280	90	—	3·12	44·5	—
,,	472-fold	16,000	5,400	—	—	2·96	—	—
,,	1670-fold	57,000	19,000	1,250	—	3·00	45·5	—
Dog pancreas	0	310	15,500	—	—	0·02	—	—
,,	97-fold	30,000	9,100	520	7,200	3·29	57·5	4·15
,,	1930-fold	600,000	181,000	10,800	150,000	3·31	55·5	4·00

* Acetylcholine chloride (Hoffmann-La Roche) was used in all experiments.

† Since the tributyrin at our disposal contained some inhibitory substance, it was purified by fractionated distillation in a high vacuum (0·003 mm. Hg). The pure substance distilled at b.p. 97°–98° (bath temperature 135–140°), $n_D^{26°} = 1·4340$.

(*b*) *The effect of eserine on choline and non-choline ester hydrolysis*

Easson & Stedman [1937], experimenting with various blood sera, found that about a thousand times the amount of eserine sufficient for the complete inhibition of choline ester hydrolysis only partially abolished the hydrolysis of non-choline esters.

We are able to confirm these observations, and agree with Stedman's interpretation that a special enzyme, esterase, capable of hydrolysing non-choline esters and scarcely sensitive to eserine, is present in certain untreated sera. As such sera (guinea-pig, horse) and crude tissue extracts (dog pancreas) contain far greater quantities of esterase than of 'cholinesterase' the action of the former would obviously conceal the inhibitory effect of eserine on the activity of the latter towards non-choline esters.

We decided, therefore, to examine the effect of eserine on the non-choline ester hydrolysis brought about by our purified extracts, from which we believed the esterase had been removed (see section 1, *a*).

Table 2 shows that small amounts of eserine inhibit the hydrolysis of acetylcholine and of non-choline esters to practically the same extent. Apart from confirming the absence of esterase, these experiments make it highly improbable that two enzymes—the one acting on choline esters, the other on non-choline esters—are present in our purified preparations, thus supporting our hypothesis that the 'cholinesterase' in serum and pancreas is a non-specific enzyme.

(*c*) *The action of serum and tissue extracts on mixed substrates*

If tissue extracts or sera contained two enzymes capable of hydrolysing substrates A and B respectively, addition of the activities of both enzymes should occur in the presence of the two substrates, provided the substrate concentrations were optimal and substrate A did not inhibit the hydrolysis of substrate B and vice versa. The latter point has already been dealt with by Stedman [1935], who found that choline esters do not inhibit appreciably,

Table 2. *The effect of eserine on the hydrolysis of acetylcholine and tributyrin by purified 'cholinesterase' preparations*

Source of 'cholinesterase' preparation	Eserine concentration *M*	Hydrolysis (in μl. CO_2 evolved in a 5 min. period) of: Acetylcholine (0·003 *M*)	Hydrolysis (in μl. CO_2 evolved in a 5 min. period) of: Tributyrin (0·003 *M*)	% inhibition of hydrolysis of: Acetylcholine	% inhibition of hydrolysis of: Tributyrin
Horse serum (Q_{Ach} 16,000)	0	66·0	22·5	—	—
	10^{-8}	42·5	15·5	36	31
	10^{-7}	9·0	2·5	86	89
	10^{-6}	0	0	100	100
Dog pancreas (Q_{Ach} 600,000)	0	39·5	12·5	—	—
	10^{-8}	20·5	6·0	48	52
	10^{-7}	7·0	2·5	82	80
	10^{-6}	3·5	1·5	91	88

if at all, the hydrolysis of tributyrin. Experimenting with various blood sera, Easson & Stedman [1937] observed that addition of activities did occur in the presence of two substrates (tributyrin and butyryl choline), the addition being almost complete in the case of guinea-pig serum, partial in the case of horse serum and less pronounced in the case of human

serum. Once more we are able to confirm these results for untreated sera and crude tissue extracts, and we again agree with Stedman's interpretation that the addition of activities is caused by a second enzyme, esterase, abundant in the sera of guinea-pig and horse.

If, however, purified preparations from which the esterase has been removed (see Section 1, *b*) are allowed to act on a mixture of two substrates, a choline ester (acetylcholine) and a non-choline ester (tributyrin), no additive effect can be observed (Table 3).

Table 3. *The hydrolysis of mixed substrates by purified 'cholinesterase' preparations*

Source of 'cholinesterase' preparation	μl. CO_2 evolved in 5 min. from Acetylcholine (0·03 *M*)	Tributyrin (0·03 *M*)	Acetylcholine (0·03 *M*) + tributyrin (0·03 *M*)
Serum (Q_{Ach} 16,000)	105	34	100
Pancreas (Q_{Ach} 600,000)	42	13	42

This result is consistent with our assumption that the hydrolysis of choline esters in serum and certain tissues is brought about by an enzyme also capable of hydrolysing non-choline esters.

We have presented and discussed three types of evidence in support of our hypothesis that one enzyme is the agent for the hydrolysis of choline and non-choline esters in our purified 'cholinesterase' preparations.

(*a*) the unaltered $Q_{Ach}/Q_{non\text{-}ChE}$ ratio within the range of hundred-fold to two thousand-fold purification;

(*b*) the identical inhibitory effect of eserine on choline ester and non-choline ester hydrolysis, when 'cholinesterase' alone is present;

(*c*) the complete lack of addition of activities when a choline and a non-choline ester are hydrolysed together.

The concurrence of these three different lines of evidence can, we believe, be accepted as conclusive proof that the 'cholinesterase' in horse serum and dog pancreas is a non-specific enzyme.

The term cholinesterase, suggestive of substrate specificity, should, therefore, not be applied to the non-specific enzymes in sera and certain tissues and might be replaced by the term pseudo-cholinesterase.

2. THE SPECIFIC OR TRUE CHOLINESTERASE

Extending our studies on the specificity of cholinesterase to other mammalian tissues, we discovered in red blood cells and brain tissue of various species a true cholinesterase, acting exclusively on choline esters.

Cholinesterase in red blood cells. Table 4 shows that laked red blood cells are capable of hydrolysing both acetylcholine and non-choline esters, such as tributyrin and methyl butyrate. By a simple method of purification (see Appendix) blood cell extracts were obtained, displaying increased activity per unit weight towards acetylcholine, while being completely inactive towards tributyrin and methyl butyrate (Table 4).

Table 4. *The activity, before and after purification, of cholinesterase in red blood cells towards choline and non-choline esters*

Source of red blood cells	Q_{Ach} Before purification	Q_{Ach} After purification	Q_{Tb} Before purification	Q_{Tb} After purification	Q_{Mb} Before purification	Q_{Mb} After purification
Human	23·0	440	5·4	0	2·5	0
Sheep	11·5	183	6·5	0	2·0	0
Horse	7·0	178	2·6	0	—	—
Ox	19·5	340	3·0	0	—	—

Substrate concentration 0·003 *M*.

These experiments, we believe, justify the conclusion that a specific cholinesterase is present in the red blood cells of various mammals.

Cholinesterase in brain. Brain tissue (mouse, dog), which effectively hydrolyses acetylcholine, exhibits some activity towards tributyrin and tripropionin, but apparently none towards methyl butyrate (Table 5). Unfortunately, brain cholinesterase does not lend itself readily to purification, and has not yet been separated from the other esterases present in brain tissue.

Nevertheless, the effect of eserine on the choline and non-choline ester hydrolysis, brought about by brain tissue, and the action of brain tissue on mixed substrates, enable us to arrive at a definite conclusion regarding the specificity of the cholinesterase in brain. Similar experiments with certain other unpurified esterase preparations, e.g. guinea-pig serum or crude pancreas extracts, would have been inconclusive, since the hydrolysis of non-choline esters by these preparations, far exceeding the hydrolysis of acetylcholine, would have concealed the presence of pseudo-cholinesterase (see Section 1, *b*). No such interference, however, occurs in experiments with crude brain preparations, owing to the fact that brain tissue, with rare exceptions, hydrolyses choline esters far more readily than non-choline esters.

(*a*) *The effect of eserine on choline and non-choline ester hydrolysis brought about by brain tissue*

Table 5 shows that an amount of eserine which almost completely inhibits the hydrolysis of acetylcholine has practically no effect on the hydrolysis of tributyrin. Indeed, the concentration of eserine must be increased more than hundred-fold before any appreciable inhibition is observed.

Table 5. *Effect of eserine on the hydrolysis of choline and non-choline esters by cholinesterase in brain*

The brain tissue was ground and suspended in about 5 volumes of distilled water, centrifuged, and the precipitate containing the cholinesterase was again suspended in the same volume of water. 0·2 ml. of this suspension was employed in all experiments except the last set (Nucleus caudatus) where 0·05 ml. was used).

Source of cholinesterase	Molar concentration of eserine	μl. CO_2 evolved in 10 min. from Acetylcholine	Tributyrin	Methylbutyrate	Tripropionin
Mouse brain (whole)	0	53·0	34·0	0	—
	10^{-6}	7·0	34·0	—	—
	10^{-5}	0	34·0	—	—
	10^{-4}	0	36·0	—	—
Dog brain (grey matter, cortex)	0	27·5	34·5	0	—
	10^{-6}	0	—	—	—
	10^{-5}	0	33·0	—	—
	10^{-4}	0	35·0	—	—
Dog brain (white matter)	0	62·0	26·0	—	—
	10^{-6}	4·0	24·5	—	—
	10^{-5}	0	23·5	—	—
	10^{-4}	0	20·0	—	—
Dog brain (grey matter, Nucleus caudatus)	0	92·0	—	—	9·0
	10^{-7}	8·0	—	—	8·0
	10^{-6}	0	—	—	8·0

If one enzyme were responsible for the hydrolysis of acetylcholine and tributyrin—as was the case in experiments with purified enzyme preparations from serum and pancreas (Table 2)—small amounts of eserine would inhibit the hydrolysis of both esters to the same extent. The fact that a concentration of eserine, which completely inhibits the hydrolysis of acetylcholine, has little or no effect on the hydrolysis of tributyrin, shows that the enzyme responsible for the hydrolysis of acetylcholine has practically no share in the hydrolysis of non-choline esters and can, therefore, be regarded as a specific cholinesterase.

(*b*) *The action of brain tissue on mixed substrates*

Table 6 gives the results of two typical experiments in which a choline ester (acetylcholine) and a non-choline ester (tributyrin) are added to a suspension of ground brain tissue both separately and together. When the two substrates are added together, the hydrolytic action of the brain tissue is exactly equal to the sum of its separate actions on the choline and the non-choline ester respectively.

If a pseudo-cholinesterase, i.e. an enzyme capable of hydrolysing both substrates, were present, the activity of the brain tissue towards the substrate mixture would be less than the sum of the activities which were observed when the substrates were added separately.

The fact that complete addition of activities takes place, reveals the presence of at least two distinct ester-hydrolysing enzymes, and excludes the existence of a pseudo-cholinesterase in brain tissue.

Table 6. *The hydrolysis of mixed substrates by suspensions of ground brain tissue*

The suspensions were prepared as described in Table 5.

Cholinesterase preparation	μl. CO_2 evolved in 5 min. from Acetylcholine (0·003 *M*)	Tributyrin (0·003 *M*)	Acetylcholine (0·003 *M*) + tributyrin (0·003 *M*)
Dog brain (grey matter, nucleus caudatus)	68	6	74
Mouse brain (whole)	36	12	48

The above experiments prove, we believe, that the cholinesterase in the brain of mammals is a specific enzyme.

The true cholinesterase, found in brain tissue and red blood cells, may also be present in other tissues not yet investigated. Even in tissues such as pancreas, in which, according to our experiments, the hydrolysis of acetylcholine seems to be entirely due to the action of a pseudo-cholinesterase, the presence of small amounts of true cholinesterase cannot be excluded.

3. THE ACTIVITY OF CHOLINESTERASE AND PSEUDO-CHOLINESTERASE AT VARIOUS CONCENTRATIONS OF ACETYLCHOLINE

The pseudo-cholinesterases in human and horse serum exhibit their maximum activities at acetylcholine concentrations of about 1·0 and 0·3 % respectively [Glick, 1937; Stedman, 1935], concentrations far higher than could be expected to occur in the blood stream. At relatively low concentrations of acetylcholine, still too high, however, to be compatible with life, the pseudo-cholinesterases display only a fraction of their maximum activity. If, for instance, 300 mg. of acetylcholine are added to 100 ml. of horse serum (38°, *p*H 7·4), we find that the hydrolysis of 3 mg. of the substrate is a matter of no more than 20 sec.; if, on the other hand, only 3 mg. of acetylcholine are added to 100 ml. of serum, 3 min. are required for the hydrolysis of the same amount of substrate.*

Unlike pseudo-cholinesterase, the true cholinesterase, found in brain tissue and red blood cells, is far more effective at low concentrations of acetylcholine; indeed, the lower the substrate concentration, the greater the activity of the specific cholinesterase (Tables 7 and 8). It may be noted that recently, Alles & Hawes [1940], continuing the work of Galehr & Plattner [1928], made the interesting observation that human red blood cells,

* The activity-substrate concentration curve of the pseudo-cholinesterase from dog pancreas is almost identical with the curve of the pseudo-cholinesterase from horse serum.

which according to our experiments contain the specific cholinesterase, were responsible for most of the action of whole blood on acetylcholine, when the substrate was present at low concentrations. They also found that high concentrations of acetylcholine inhibited the action of cholinesterase in human red blood cells. At the same time they noted differences between the serum cholinesterase, which we have found to be a pseudo-cholinesterase, and the cholinesterase present in red blood cells.

Table 7. *Hydrolysis of acetylcholine by cholinesterase from red blood cells*

The horse blood cells were purified in the manner described in the Appendix.

Concentration of acetylcholine mg. %	μl. CO_2 evolved in the 2 min. period following addition of substrates to haemolysed blood cells of			
	Man	Sheep	Horse	Ox
500·0	10·0	13·5	6·0	12·5
50·0	17·0	25·0	19·5	—
25·0	—	30·0	20·5	28·0
2·5	19·0	—	24·0	32·0

Table 8. *Hydrolysis of acetylcholine by cholinesterase in brain*

The brain suspensions were prepared as described in Table 5.

Concentration of acetylcholine mg. %	μl. CO_2 evolved in the 2 min. period following addition of substrates to suspensions of		
	Mouse brain (whole)	Dog brain (grey matter, cortex)	Dog brain (whole)
500·0	5·0	4·5	4·0
50·0	—	11·0	8·0
25·0	13·0	—	11·0
12·5	19·0	14·0	—
5·0	21·0	16·5	—
2·5	22·5	18·5	—

Since it seems reasonable to assume that usually low concentrations of acetylcholine occur in living organisms, it is most likely that the destruction of acetylcholine, wherever it takes place rapidly, is brought about mainly, if not exclusively, by the specific cholinesterase.

SUMMARY

Experiments performed with highly purified esterase preparations capable of hydrolysing acetylcholine, show that the 'cholinesterase' present in blood serum and certain tissues is a non-specific enzyme, catalysing the hydrolysis not only of choline esters but of non-choline esters as well.

A specific cholinesterase, however, acting exclusively on choline esters, was found in the brain tissue and the red blood cells of some mammals.

The non-specific 'cholinesterase', purified from horse serum and dog pancreas, exhibits its maximum activity at high concentrations of acetylcholine (above 300 mg./100 ml.) and only slight activity at low substrate concentrations. The reverse is true for the specific cholinesterase, found in brain tissue and red blood cells: while inhibited by high concentrations of acetylcholine, it displays its maximum activity at low substrate concentrations (below 3 mg./100 ml.).

It is proposed to name the non-specific enzyme pseudo-cholinesterase, and to reserve the term cholinesterase for the specific enzyme.

APPENDIX

Method for the separation of cholinesterase from other esterases in red blood cells

10–15 ml. of freshly drawn oxalated blood (human, horse, sheep, ox) are centrifuged, and the plasma is discarded. The red blood cells are washed twice with about 30 ml. of physiological saline solution. A layer of white blood cells which forms on top of the red blood cells after centrifuging, is removed as completely as possible, by suction with a capillary.

To 5 ml. of the washed red blood cells 45 ml. of distilled water are added. When complete haemolysis has taken place, the solution is shaken thoroughly with 1 g. of kieselguhr (B.D.H., acid-washed). The mixture, including all the kieselguhr, is then filtered through filter paper on a Büchner funnel, 7 cm. in diameter. The filtrate, containing practically all the haemoglobin, is poured back, until it is absolutely clear, and is then discarded. The almost-dry filter cake is scraped from the filter paper, thoroughly shaken with 50 ml. of distilled water and the filtration repeated as previously described.

The filter cake is again scraped from the filter paper and suspended in 5 ml. N/1000 NaOH. The suspension is shaken thoroughly for a few minutes and then centrifuged at high speed.

The slightly turbid supernatant fluid, orange-red in colour, is 20–30 times as active (per unit dry weight) as the original blood cells towards acetylcholine, and inactive towards non-choline esters, such as tributyrin.

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Studies on Cholinesterase

2. A METHOD FOR THE PURIFICATION OF A PSEUDO-CHOLINESTERASE FROM DOG PANCREAS

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Many papers have been published on the properties and distribution of cholinesterase in the animal body but few attempts have been made at its purification. Stedman & Stedman [1935] purified the enzyme from horse serum 50 to 100 times; McMeekin [1939], using a different method, slightly increased the degree of purity. In this laboratory preparations about 20 times as active as Stedman's were procured from horse serum, but the yield was very low and the method not suitable for operations on a larger scale.

The only previous attempt to purify cholinesterase from tissues was made by Nachmansohn & Lederer [1939], who obtained aqueous extracts from the electric organ of the *Torpedo vulgaris* which were about three times as active as Stedman's purest preparations.

For the purification of tissue cholinesterase, to be described in this paper, dog pancreas was chosen, as it was known to contain large amounts of the enzyme [Marnay, 1938]. The pancreas of larger animals such as horse, ox and hog were tested for cholinesterase activity, but were so much less active than dog pancreas that purification was not attempted.

The method of purification is relatively simple. Only two chemicals, $(NH_4)_2SO_4$ and kieselguhr, are used in the process, which consists of four main steps: (1) extraction of the enzyme with water; (2) fractionation with $(NH_4)_2SO_4$; (3) adsorption on to kieselguhr, and (4) elution with ammonium sulphate.

The final preparation, 1 mg. of which hydrolyses about 1·2 mg. of acetylcholine per sec., is approximately 2000 times as active per unit dry weight as its source and 100–200 times as active as the purest preparations described by McMeekin or Stedman.

The purified enzyme proved to be a pseudo-cholinesterase [Mendel & Rudney, 1943], since it attacked a variety of non-choline esters and hydrolysed acetylcholine with maximum speed at a comparatively high substrate concentration (about 0·02 *M*).

EXPERIMENTAL

Estimation of cholinesterase activity

The activity of the enzyme is measured manometrically by the Warburg method, first adopted for the measurement of cholinesterase activity by Ammon [1933]. An aqueous solution of the enzyme preparation (0·1 ml. or less) is placed in the main compartment of a Warburg flask and brought to a volume of 1·9 ml. with a solution of 0·025 *M* $NaHCO_3$, previously saturated with 5 % CO_2 in N_2. The flask is attached to the manometer and filled with the above-mentioned gas mixture, in order to maintain the enzyme solution at a constant *p*H (7·4). 0·1 ml. of a 10 % aqueous solution of acetylcholine chloride (Hoffmann la Roche) is tipped from the side bulb into the main compartment after temperature equilibrium at 37·5° has been attained.

1 m.-mol. of acetylcholine, when hydrolysed by the enzyme into choline and acetic acid, evolves 1 m.-mol. of CO_2 from the $NaHCO_3$. The amount of CO_2 in μl. evolved in 1 hr. by 1 mg. dry weight of the enzyme preparation, is referred to as the acetylcholine quotient (Q_{ACh}).

METHOD OF PURIFICATION

1. *Extraction*

The pancreas is removed from 4 or 5 dogs immediately after electrocution, rinsed with cold water, and freed from fat, mesentery and visible blood vessels. The tissue, weighing about 100 g., is cut into small pieces, finely minced (an electric mixer, 'Whizmix', was found very efficient for mincing and mixing the tissue with water) and extracted with 9 parts of cold distilled water. Extraction is allowed to continue for at least 5, but not longer than 20 hr., in the refrigerator (about +2°); the mixture is then strained through a coarse sieve (18 mesh) to remove fibrous tissue. The Q_{ACh} of the strained suspension varies from 200 to 400, the higher values generally being obtained during July, August and September.

2. *Fractionation with ammonium sulphate*

A. *Precipitation at 0·48 saturation.* The strained suspension is gradually brought to 0·48 saturation with an $(NH_4)_2SO_4$ solution (sat. at 20°) by adding 2/3 of the required $(NH_4)_2SO_4$ at once and the remainder 2 hr. later: this prevents any of the albumin fraction from being carried down prematurely with the bulky precipitate. Throughout this procedure, and for 3 or 4 hr. afterwards—the time required for

complete flocculation—the mixture is allowed to stand in the refrigerator. At this stage of purity the enzyme preparation can be kept in the cold for weeks without loss of activity.

The flocculent suspension is then centrifuged in a high speed centrifuge (a Sharples Super Centrifuge run by air pressure) and the precipitate is discarded.* The active (0·48 sat.) fluid is brought to 0·85 sat. with solid $(NH_4)_2SO_4$ in order to concentrate the enzyme by precipitation. The mixture, having been kept in the cold for at least 3 hr., is repeatedly filtered through filter paper on a Büchner funnel by suction, until the filtrate is clear.

The active filter cake is scraped from the filter paper and thoroughly mixed with a quantity of water equal to 1/20–1/10 of the original volume of the extract. The mixture is centrifuged to eliminate coarse insoluble material; a floating layer of fat, which may have formed, is removed as completely as possible. The turbid supernatant fluid is then dialysed in cellophane bags against running tap water (below 15°) for at least 16 but not longer than 20 hr. An inactive precipitate which forms during dialysis is removed by centrifuging.

The dialysed enzyme solution is again brought to 0·85 sat. with solid $(NH_4)_2SO_4$ and filtered as described above. The filter cake is dissolved in the smallest possible amount of water and dialysed against running tap water for 20 hr. After dialysis any precipitate is centrifuged off and the brownish coloured enzyme solution is dried in vacuo over $CaCl_2$ at a temperature not exceeding $+2°$. At this stage the Q_{ACh} ranges from 8000 to 12,000. Since the dried active material is stable when kept on ice, it can conveniently be collected and stored at this point.

When a sufficient quantity of the dried preparation (3–4 g.) has been accumulated, it is dissolved in about 10 parts of cold distilled water. Much insoluble inactive material is removed by centrifuging, and the enzyme solution is dialysed in the refrigerator for 36 hr. against frequently changed distilled water. After dialysis the Q_{ACh} is about 20,000.

B. *Precipitation at 0·5 saturation.* An equal volume of sat. $(NH_4)_2SO_4$ is added to the dialysed enzyme preparation, and the mixture is kept in the refrigerator for about 1 hr. The resulting flocculent precipitate is inactive and should be removed by centrifuging.

C. *Precipitation at 0·52 saturation.* By addition of more saturated $(NH_4)_2SO_4$ solution, the active 0·5 sat. fluid is brought to exactly 0·52 sat. and is then kept at 37° for 15 min. An inactive precipitate forms and is removed by centrifuging. The active fluid is again concentrated by precipitation with solid $(NH_4)_2SO_4$ (0·85 sat.) and is filtered as before. The filter cake is dissolved in as small a volume of water as possible and dialysed against running water (below 15°) for 20 hr. At this stage the preparation has a Q_{ACh} of approximately 40,000. It may be dried in vacuo at a temperature not exceeding 2° and stored in the cold for about 2 months without loss of activity.

3. *Adsorption on to kieselguhr*

When at least 0·5 g. of the dried enzyme material with a Q_{ACh} of about 40,000 has been accumulated, it is dissolved in about 20 parts of cold water and dialysed against frequently changed distilled water in the refrigerator for 36 hr. After dialysis, the brownish coloured liquid is shaken for 10 min. with the least amount of kieselguhr* which will adsorb about 95 % of the active material. The kieselguhr suspension is cooled in ice-water and centrifuged for a few minutes. The supernatant liquid, containing the greater part of the brownish coloured inactive material, is discarded. The kieselguhr is washed with cold distilled water and then centrifuged; this is repeated several times until the water has become colourless. Very little active material is lost in the course of this procedure.

4. *Elution*

A cold 0·1 sat. $(NH_4)_2SO_4$ solution, equal in volume to the enzyme solution before adsorption, is added to the kieselguhr. The suspension, while being cooled in ice-water, is stirred mechanically by a glass rod for 10–15 min. It is then centrifuged for a very short time. The resulting supernatant liquid contains 40–60 % of the adsorbed active material and the yield can be increased to about 75 % by a second elution.

The combined clear eluates are brought to 0·85 sat. with solid $(NH_4)_2SO_4$ and are kept in the cold for at least 6 hr. A flocculent precipitate forms and the suspension is filtered repeatedly through filter paper on a small Büchner funnel (diameter *ca.* 2 cm.) by suction until the filtrate is perfectly clear and the filter cake almost dry. The filter paper with the adhering layer of precipitate is placed in a small beaker containing not more than 5 ml. of ice-cold distilled water. Unless a fairly concentrated solution of the enzyme material is prepared, considerable loss of activity may occur during dialysis. The active material, which dissolves readily, is transferred to a cooled centrifuge tube, and the liquid absorbed by the filter paper is squeezed out and added. The

* The esterase is associated with both the globulin and albumin fractions of the pancreas proteins, but since it was found very difficult to purify the globulin part, only the albumin fraction (containing about 1/2 of the esterase activity) was used for further purification.

* About 3 g. of kieselguhr 'Brocades-Stheeman (Amsterdam)' are required for 50 ml. of the enzyme solution. Other brands of kieselguhr, such as B.D.H. kieselguhr, white, are suitable but not quite as efficient.

solution, having been cooled, is centrifuged for 2 or 3 min. to remove any traces of filter paper, and should then be dialysed for 2 days in the refrigerator against frequently changed distilled water. An inactive precipitate may form which can be eliminated by centrifuging. The enzyme solution is now colourless and has a Q_{ACh} of 400,000–600,000.

The yield of this purified preparation amounts to about 20% of the original activity of the pancreas, but is much greater (40%) when calculated on the basis that only the albumin fraction is used for purification.

Since enzyme preparations with a Q_{ACh} above 10,000 are adversely affected by salts and dilution with water,* they should be diluted, and their activity measured, in a solution of 0·25% gum acacia.

* Effects of salts and dilution with water will be discussed in a subsequent paper dealing with the properties of the purified enzyme.

SUMMARY

A simple method is described by which a tissue esterase, capable of hydrolysing acetylcholine, may be purified from dog pancreas. The final preparation, 1 mg. of which hydrolyses about 70 mg. of acetylcholine per minute, is 2000 times as active per unit dry weight as the original material and about 15,000 times as active as horse serum.

The enzyme is a pseudo-cholinesterase, since it hydrolyses esters other than those of choline, and splits acetylcholine with maximum speed at a substrate concentration (0·02 *M*) far above physiological range.

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Methods for the Purification of Tomato Bushy Stunt and Tobacco Mosaic Viruses

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Since we first described methods for making liquid crystalline preparations of tobacco mosaic virus [Bawden & Pirie, 1937] and crystalline preparations of tomato bushy stunt virus [Bawden & Pirie, 1938], we have made preparations of both viruses by many other methods. As a result, we can now describe simpler and better procedures. Other workers have used differential ultracentrifugation; because of the infrequency with which stable substances of high molecular weight seem to occur in normal leaves, this method of preparation is undoubtedly of great value. The handling of large volumes of sap in the ultracentrifuge, however, is laborious, and many laboratories where plant viruses are studied do not have this equipment. Methods of preparation that are trustworthy and involve only ordinary centrifuges may therefore be of more general use.

Tomato bushy stunt virus

In our earlier method the sap was heated to 60° to coagulate the normal leaf protein, and if the preparation is needed solely for a study of its serological or physical properties this is still probably the simplest procedure. However, heating to this extent greatly reduces the infectivity of the virus [Smith, 1935; Stanley, 1940], apparently without affecting its other properties appreciably, and more infective preparations can be made in other ways. The method described below is the most satisfactory we have found, but with small quantities of sap, precipitation with alcohol, as used by Pirie, Smith, Spooner & McClement [1938] for tobacco necrosis viruses, is also suitable and quicker.

Preparation. Leaves and stems of infected tomato plants are put through a domestic meat mincer with $\frac{3}{16}$ in. holes in the plate, and sap is expressed from the mince by hand through a bag of thin, closely woven cloth such as madapollam. For each 100 ml. of sap expressed, 30 ml. of a 4% solution of anhyd. Na_2HPO_4 is added to the residue in the bag; when this has soaked in, the residue is again put through the mincer and the sap is expressed as before. The two extracts are mixed, centrifuged and the precipitate discarded. The supernatant fluid is usually clear and brown,

but it sometimes has a greenish tinge; this can be removed by adding some more phosphate solution. $(NH_4)_2SO_4$ is added (280 g./l.), and after it has all dissolved the fluid is left for about 12 hr. at room temperature. The precipitate is then centrifuged off, and suspended evenly in a volume of water equal to about one-tenth of the original sap. The fluid is then centrifuged and the precipitate extracted twice or thrice more with smaller volumes of water. The three supernatant fluids are mixed, acetic acid is added to bring the *p*H to 4, the bulky precipitate is centrifuged off, and extracted twice with water. All subsequent operations are carried out at *p*H values between 4 and 4·5.

$(NH_4)_2SO_4$ is added to the mixed acid fluids until there is a definite turbidity; it is convenient to add the solid until about 15 g./100 ml. have been added and then to continue with a saturated solution. Within a few hours at room temperature a precipitate separates from the turbid solution. This can be centrifuged off after about 6 hr. and contains most of the virus. After standing for a few days, however, or after the addition of a little more salt, a further precipitate containing a little virus may separate from the supernatant fluid. This is most conveniently worked up separately. The precipitate is extracted two or three times with dilute acetate buffer so that the volume of the combined extracts is about one-hundredth of that of the original sap. The extract should be colourless or light brown, and only slightly opalescent. At this stage, if old plants have been used, the fluid may still be dark brown; it should then be reprecipitated with $(NH_4)_2SO_4$ and the precipitate, suspended in the minimum amount of water, should be dialysed. As dialysis proceeds a heavy brown precipitate separates; this is centrifuged off, washed once and the fluid used for the extraction of precipitates obtained at a later stage. Saturated $(NH_4)_2SO_4$ solution is now added drop by drop with thorough mixing at a temperature between 20 and 25° until the fluid becomes permanently turbid. Solutions containing more than 1% of virus will begin to crystallize when the $(NH_4)_2SO_4$ concentration has been raised to 10%, but 15% will be needed if the virus concentration is as low as 1 g./l. A precipitate separates after 1–2 min. but dissolves again almost completely when the mixture is cooled to 0°. After 2–3 hr. at 0° any material that remains undissolved is removed by centrifuging at 0°. This can be done in a refrigerated centrifuge or by putting the centrifuge inside a refrigerator. It can, however, conveniently be done in an ordinary machine at room temperature by use of the device illustrated in Fig. 1. A wooden block and lid are turned to fit easily inside the 250 ml. bronze bucket of an International centrifuge and a hole is bored in the block to contain a 50 ml. glass or metal centrifuge tube. If the upper surface of the lid lies a few mm. below the rim of the bronze bucket there is no risk of its being blown off when the centrifuge is spinning. The virus from 1 l. of sap can conveniently be held in four $3 \times \frac{3}{8}$ in. test-tubes, and these are carried, surrounded by ice and water, in the 50 ml. tube. In the model that we use the wooden walls and lid are 12 mm. thick, and some ice remains unmelted after 45 min. centrifuging at 2000 r.p.m. The precipitate can generally be packed tightly by 15 min. centrifuging; if so much $(NH_4)_2SO_4$ has been added that an appreciable amount of virus has also precipitated, it is well to extract the precipitate with water and repeat the precipitation at 0° on the extract.

At this stage the supernatant fluid should be clear and colourless; when left at 0° crystallization will start in a few hours, but if it is allowed to warm up there will be rapid separation of an amorphous product. More virus can be separated in the first fraction if, after a few days, another drop of saturated $(NH_4)_2SO_4$ solution is added for each ml. of fluid. Most of the crystals form on the walls of the tube and, after 4–7 days, the mother liquor can be poured off without centrifuging. If the addition of $(NH_4)_2SO_4$ has been gauged accurately only a tenth of the virus will remain in the mother liquor; this precipitates when the fluid is allowed to warm up and can be centrifuged off, redissolved and crystallized. The crystals do not dissolve completely unless they are left in water for about an hour,

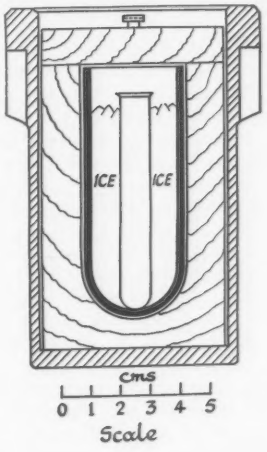

Fig. 1.

and a useful separation from residual contaminants can be effected by washing them quickly. When they have dissolved, any insoluble material is removed, by centrifuging, either immediately or after dialysis. Crystallization seems to proceed equally satisfactorily at any *p*H between 3 and 7, but the removal of traces of denatured virus and normal leaf protein is easiest at about *p*H 4·5.

One variant of this method may on occasion be useful. The addition of enough acid to bring the *p*H of the original sap to 4·5 removes more normal protein than the addition of phosphate and leads to no greater loss of virus. It does not, however, remove so much of the Ca from the sap, and this, if not removed, precipitates with the $(NH_4)_2SO_4$ and may interfere with the subsequent extraction of the virus from the first $(NH_4)_2SO_4$ precipitate. If the leaves that are being used in a virus preparation happen to have a low Ca content, there may be greater advantages in getting rid of the normal protein than in getting rid of the Ca, and this variation may make the preparation go more smoothly.

The C, H and N content of a virus preparation gives but little information about its purity; the P content, on the other hand, is a useful guide. In our earlier paper we gave 1·3–1·5% as the range within which the P contents of our preparations fell. In the more extended series of analyses that has since been carried out, on preparations dried at room temperature *in vacuo* over P_2O_5, we find that preparations containing less than 1·4% can always

be further fractionated. Under the conditions used by us the P method of Kuttner & Lichtenstein [1932] has a probable error of 5%. With this uncertainty we look upon 1·5% as the most probable value for the P content. An impure preparation of bushy stunt virus is likely to have a low P content, but, as we have already pointed out [Bawden & Pirie, 1938], its carbohydrate content will probably be high. The precipitate that separates from most preparations on prolonged dialysis at about *p*H 4·5 invariably has a high carbohydrate content, and it is rare to find a carefully purified preparation that contains as much as 7% of carbohydrate estimated by the orcin method [Pirie, 1936] using glucose as a standard. The most usual value is 6%. It is not possible to assess the significance of the difference between this figure and the range 11·4 to 7·1% given for the carbohydrate content of centrifugally isolated virus by Stanley [1940], for he does not mention the method of estimation used, and the different methods of carbohydrate estimation give results that are not necessarily comparable.

The only difference we have found between preparations made by the method described in this paper and by that described earlier is that the former are more infective. In appearance, crystallizability, serological activity, analytical composition and sedimentation constant, they are indistinguishable. Recent measurements on preparations made by both methods give an $S_W^{20'} = 130 \times 10^{-13}$. The discrepancy between this figure and that given by McFarlane & Kekwick [1938] is discussed in the Addendum to Bawden & Pirie [1943].

Table 1. *Comparison of activity of bushy stunt virus prepared by two methods*

Test	Method	Serological titre	Infectivity Av. no. of lesions per leaf at 10^{-4}	Infectivity Av. no. of lesions per leaf at 10^{-5}
1	Heating	1/600,000	35	9
	No heating	1/600,000	105	45
2	Heating	1/600,000	26	4
	No heating	1/600,000	96	40

In Table 1 purified preparations made by the two methods are compared, and it will be seen that virus made by the method described in this paper is at least 10 times as infective as virus made by the method involving heating. It is more difficult to be certain that the method causes no loss of infectivity. Comparisons between the infectivity of sap and the purified virus suggest that there is no loss, but it is well known that the constituents of sap can affect the number of lesions produced. The extent of this depends at least in part on the ratio of virus to normal plant constituents. This is illustrated in Table 2; during one purification the removal of contaminants has given an apparent increase in infectivity whereas in a second, with much more infective sap, it has not. To get the most highly infective preparations, sap should be worked up immediately, for, as we have already shown [Bawden & Pirie, 1940], during ageing *in vitro* the virus loses infectivity in much the same way as when heated.

Table 2. *Comparison of activity of sap and purified bushy stunt virus preparations*

Preparation	Serum precipitation end-point	Av. no. of lesions per leaf at 1:20	Av. no. of lesions per leaf at 1:200
Preparation 1:			
Sap from stalk and leaves	1:12	44	7
Virus preparation diluted to original vol. of sap	1:12	67	20

Preparation	Serum precipitation end-point	Av. no. of lesions per leaf at 1:10	Av. no. of lesions per leaf at 1:100	Av. no. of lesions per leaf at 1:1000
Preparation 2:				
Sap from stalk and leaves	1:160	186	86	8
Virus preparation diluted to original vol. of sap	1:120	188	86	6

The virus content of the leaves from infected tomato plants is at least five times as great as that of the stems; the inclusion of stems in the preparation therefore has only a small effect on the yield and increases greatly the labour of mincing. The mincing is easier if the leaves have been frozen beforehand, but this freezing has no other advantage. We have, however, no evidence that it causes any loss of virus, for the salts and other constituents of sap protect the virus from the inactivation that takes place when virus solutions in water are frozen [Bawden & Pirie, 1938]. This phenomenon will be dealt with more fully in the following paper.

We have generally used tomato plants as a source of the virus and the yields have varied from 3 to 100 mg./l. of sap. The highest yields are obtained from young seedlings infected in the winter and the lowest from older plants infected in the summer. Purification is also easier from plants growing during the winter, as the normal plant constituents are then less troublesome to remove. We have also made preparations from *Datura stramonium* and *Nicotiana glutinosa*. *D. stramonium* is recommended by Stanley [1940] as giving a higher yield than tomato, but in our experience the sap of this plant is more troublesome to handle. The yield from *N. glutinosa*, as would be expected from a plant giving local lesions only, is very low. These yields all refer to the sap that is expressed after mincing in a domestic meat mincer. By more thorough grinding of the

leaf residue a further equal quantity of virus can be isolated. This phenomenon will be described in a later paper.

Tobacco mosaic virus

Precipitation with alcohol was one of the first steps in the method we previously described for the purification of tobacco mosaic virus. This successfully removed most of the normal leaf protein but was laborious.

In the preparations carried out during the past few years we have relied on precipitation at *p*H 3·3 to render the normal protein irreversibly insoluble. We have already stressed the aggregation that appears to accompany purification of the virus by our methods [Bawden & Pirie, 1937]; Bernal & Fankuchen [1941] suggest that this is a necessary corollary of purification and that the tendency towards linear aggregation of the particles is increased by the removal of some of the normal components of sap. Our view that preparations of tobacco mosaic virus are not homogeneous and consist of rods of equal cross-section but variable length, has received valuable support from Frampton's measurements [1942] of the lengths of virus particles measured by means of the electron microscope.

Preparation. Sap from infected plants is prepared by mincing and extraction with phosphate solution as in the method used for preparing bushy stunt virus. To each litre of fluid 250 g. of $(NH_4)_2SO_4$ is added and, after about ½ hr., the precipitate is centrifuged off; it is extracted with water until the extracts no longer have an obvious shimmer when stirred. In general four or five extractions are necessary, and the final volume of the extract is about one-quarter of the original volume of the sap. 1% NaOH is added in a fine stream with vigorous stirring to bring the *p*H to about 6·5, and the precipitation with $(NH_4)_2SO_4$ is repeated; if the supernatant is still dark brown a third precipitation is carried out. The precipitate is suspended in 10 times its bulk of water and centrifuged for 1 hr. at 3500 r.p.m., the dark brown precipitate is re-extracted with a smaller volume of water, and the virus precipitated by bringing the *p*H of the combined extracts to 3·3 by the addition of dilute HCl. After ½ hr. this precipitate is centrifuged off, washed once by mixing thoroughly with water and centrifuging again, transferred with the minimum amount of water to a dialysis sac, and dialysed against running tap water. After 24–36 hr. the *p*H will have risen sufficiently for the virus to dissolve, but at this *p*H, and in the presence of only traces of salt, most of the normal protein remains insoluble. The contents of the dialysis sac are diluted if necessary and centrifuged; centrifugation is likely to be very slow if the fluid contains more than 1% of solid. If the precipitate is bulky it should be re-extracted. This method of dissolving the acid precipitate of virus has been adopted because it avoids the risk of inactivating the virus in the small regions of high *p*H which are almost unavoidable if alkali is added directly. This dialysis also brings about a useful separation of the virus from some normal constituents of the sap.

The further treatment depends largely on the appearance of the fluid. If young tomato or white burley tobacco plants have been used it will be nearly colourless, but with older plants it will be brown. If the fluid is brown the *p*H should be adjusted to 6·5 and the $(NH_4)_2SO_4$ precipitation repeated. Pale or colourless preparations are reprecipitated with acid and dialysed thoroughly against distilled water; the dialysis sac should be shaken gently. If necessary the fluid is centrifuged and is then left undisturbed in the ice chest for a few days for the separation of the lower, liquid-crystalline, layer. With aucuba mosaic this should separate from a solution containing 2% of virus, but with the other strains 3% is generally necessary. As in preparations made by the previous method the lower layer is, from a chemical standpoint, the purer and has a higher serum precipitation end-point than the upper, but it is slightly less infective. This method of preparation gives good results with either tobacco or tomato plants.

The recovery of virus by this procedure is satisfactory and there is no evidence of any great inactivation. Thus from 1600 ml. of tomato sap 610 mg. of aucuba mosaic virus was isolated in the form of 'Bottom layer' and 435 mg. as 'Top layer', that is to say 0·66 g. was isolated for each litre of sap. The serum precipitation end-point of the sap was 1:1500. If the 0·66 g. represented complete recovery of virus this corresponds to an end-point of $2{\cdot}3 \times 10^6$, and any loss during the preparation necessitates the assumption of an even lower end-point. The 'Top' and 'Bottom' layers, on the other hand, had serum precipitation end-points of $1{:}4 \times 10^6$ and $1{:}6 \times 10^6$ respectively. For infectivity measurements it was assumed that the sap contained 0·66 g./l. and dilutions were made accordingly. In Table 3 the number

Table 3. *Average number of lesions per leaf for sap and purified aucuba mosaic virus*

	Dilution of virus		
	$1:10^5$	$1:10^6$	$1:10^7$
Sap	76	21	1
'Bottom layer'	49	15	0
'Top layer'	52	12	2

of lesions found is set out. These results are most simply explained by postulating that linear aggregation of the virus particles during purification has increased the serum precipitation titre, whereas the fall in infectivity, which would be expected to accompany such aggregation, has been masked by an apparent increase in infectivity due to the removal of normal sap constituents.

The serum precipitation end-points given above for aucuba mosaic virus are similar to the end-points 6×10^6 to 10^7, found for this strain of virus in 1937. With tobacco mosaic virus, on the other hand, we are unable to duplicate the old high values for the end-point. The strains that we handle now give precipitation end-points of about 10^6 instead of the old values of 6×10^6 and 10^7. This change is probably due to the inadvertent selection of a new strain of tobacco mosaic virus, but we have no definite evidence of this.

SUMMARY

Methods, requiring only low-speed centrifuges, are described for the purification of tomato bushy stunt and tobacco mosaic viruses.

These preparations appear to contain virus that is weight for weight as infective as that in clarified sap. There is evidence, however, that the tobacco mosaic virus particles have undergone some aggregation.

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The Inactivation of Tomato Bushy Stunt Virus by Heating and Freezing

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We have often stressed the fact that there is some uncertainty over the exact name most suitable for use when speaking of purified virus preparations. It can hardly be questioned that the specific nucleoproteins, isolated from infected plants, bear some relationship to the viruses themselves, and we have used the term viruses when referring to these proteins. The exact relationship between these proteins and the viruses as they are produced in the infected cells, however, is by no means clear.

All the viruses with which we have worked can be rendered non-infective without changing their serological reactions, and such inactive virus preparations have physical properties at present indistinguishable from those of active virus. Because of this, physical and serological tests cannot be taken as proving the homogeneity of virus preparations. With viruses such as potato '*X*' and tobacco mosaic complete loss of infectivity without loss of serological activity has been produced by X-rays, ultraviolet light, H.CHO, H_2O_2 and HNO_2; but the activity and homogeneity of purified preparations of these viruses is affected by the tendency of the particles to aggregate linearly [Bawden & Pirie, 1937; 1938*b*]. Tomato bushy stunt and tobacco necrosis viruses do not appear to aggregate in this way, but they are more easily rendered non-infective by ageing *in vitro*, moderate heating and treatment with alkali.

In the method we described for the isolation of a crystalline protein from plants suffering from bushy stunt, the sap was heated to about 60° to facilitate clarification [Bawden & Pirie, 1938*a*]. We now know that this treatment must have led to considerable inactivation, and Stanley [1940] showed that more infective preparations could be produced by differential centrifugation. More infective preparations can also be made by precipitation methods if heating is omitted. The method of isolation we describe [Bawden & Pirie, 1943] gives a crystalline product that weight for weight is as infective as the virus in clarified infective sap. At the moment this is the only test available for estimating full activity, but it is not necessarily a valid one. First, it is possible that inactivation without loss of serological activity occurs in the living plant, so that the virus in the sap is already a mixture of infective and non-infective particles. Secondly, sap may contain some inhibitor of infectivity; if this is so, some inactivation could occur during purification but pass unnoticed, as it would be balanced by the removal of the inhibitor. Until more sensitive methods for detecting activity are developed, so that it can be shown that one virus particle can cause infection, it is not likely to be possible to prove that any virus preparation is homogeneous.

The main conclusions of our earlier paper on tomato bushy stunt virus were confirmed by Stanley [1940]. There are some apparent disagreements, however, and the significance of these is discussed in this paper, which deals mainly with the effects of heating and freezing the virus. The methods of testing were similar to those described previously [Bawden & Pirie, 1938*a*].

EXPERIMENTAL

The effect of heating purified virus preparations

The actual thermal inactivation point of bushy stunt virus, i.e. the temperature at which 10 min. heating gives complete inactivation, has varied in different tests between 70 and 85°. The variation may depend partly on the concentration of the virus solution heated, but it appears chiefly to be determined by the susceptibility of the host plants used. The same inoculum may produce 10 or more times as many local lesions in one lot of test *Nicotiana glutinosa* plants as in another, and it is obvious that the term complete inactivation has no real meaning in these conditions, for whether or not infection is obtained depends first on the concentration of the inoculum and secondly on the susceptibility of the host. In general, the younger the *N. glutinosa* plants the more lesions per leaf are obtained. In moderately developed plants, there is an increase in the susceptibility of leaves from the base to the top so that the same inoculum may only give one or two lesions on a basal leaf but give 50 or 60 on leaves nearer the top. This is in striking contrast to results with tobacco mosaic virus on the same host where there is a gradient of increasing susceptibility from the top to the base. With tobacco mosaic virus the differences are less pronounced, but if solutions of the two viruses are rubbed on opposite halves of the same leaves, those at the top of the plant will often give more lesions with bushy stunt than with tobacco mosaic virus, whereas those at the base will bear great numbers if rubbed with tobacco mosaic but few or none with bushy stunt virus.

Table 1. *Effect of heat on purified bushy stunt virus made with and without previously heating infective sap*

	Virus from unheated sap		Virus from heated sap	
Temp.	Serological titre	Av. no. of lesions per leaf at 10^{-4}	Serological titre	Av. no. of lesions per leaf at 10^{-3}
Unheated	1/600,000	83	1/600,000	78
45°	1/600,000	58	1/600,000	55
50°	1/600,000	33	1/600,000	36
55°	1/600,000	16	1/600,000	19
60°	1/600,000	5	1/600,000	12
70°	1/600,000	3	1/600,000	8
80°	1/600,000	1	1/600,000	4
85°	*Nothing at 1/5000	0	*Nothing at 1/5000	0

* Tested after centrifugation; the infectivity results are expressed as the average number of lesions per leaf on *N. glutinosa*.

Influence of temperature. Table 1 shows the effect of 10 min. heating at various temperatures on two preparations of virus made in different ways, one in which the infective sap was previously heated and the other not. Both were heated as 0·1 % solutions in 0·1 *M* phosphate buffer at *p*H 6, the preparation from heated sap was tested for infectivity without further dilution and the other was diluted 1:10 so that the two were comparable in infectivity. Although loss of infectivity is only complete after heating at 85°, some infectivity is lost at temperatures as low as 50° and most of it below 60°. By contrast, the precipitation titre is unaffected by heating up to 80°, but a little heating above this is sufficient to destroy the serological reactions. This destruction is accompanied by the separation of a coagulum. Thus there is a wide range in the temperatures over which loss of infectivity can be measured, but loss of serological activity resembles other examples of protein denaturation in proceeding at a measurable rate only within a narrow temperature range. In this respect tomato bushy stunt virus resembles the tobacco necrosis viruses and differs from other viruses investigated, which lose both infectivity and serological activity *pari passu* in a narrow temperature range [Price, 1938; 1940; Bawden, 1941]. Virus preparations that have been largely inactivated by heating during the preparation lose their residual activity according to much the same law that governs the loss of activity by fully active preparations.

Influence of duration of heating. Preparations with no demonstrable infectivity can be produced by heating at temperatures well below the thermal inactivation point, but prolonged heating is necessary. Thus in *p*H 6 phosphate buffer at 50°, a 0·5 % virus solution lost half of its infectivity in 15 min. three-quarters in 1 hr. and all in 24 hr.; at 60° the solution was non-infective after 1 hr. and at 70° after 30 min. Such heated preparations do not change their appearance, they retain their full serological activity and crystallize as easily and in the same form as unheated ones.

Table 2. *The infectivity of fractions from a mixture of infective and non-infective sap*

	Av. no. of lesions per leaf at	
Virus preparation	10^{-4}	10^{-5}
1. Active virus	80	41
2. Inactive virus	0	0
3. Mixture of 1 part of no. 1 with 4 parts of no. 2	46	18
4. 1st crystalline fraction from no. 3	43	15
5. 2nd crystalline fraction from no. 3	38	16

We have attempted to fractionate preparations of partially inactivated virus by crystallization but have had no success. As many as five crystalline fractions have been made from preparations derived

from heated sap, but they all had similar infectivities. Artificial mixtures of infective and non-infective virus have also been made and subjected to fractional crystallization, without any separation. The results of one such test are given in Table 2.

Influence of p*H*. The virus is most stable around *p*H 6, but heating at any *p*H value between 4 and 9 can cause loss of infectivity without a corresponding loss of serological activity. At *p*H 4, however, denaturation occurs with much less heating than at higher values. In *p*H 6 *M*/10 phosphate buffer, 0·1 % solutions become opalescent after 10 min. at 80° and still further heating is necessary to cause precipitation. In *p*H 4 *M*/10 phthalate buffer, on the other hand, 0·1 % solutions are opalescent after 10 min. at 60° and 10 min. at 70° causes precipitation.

Heating for 10 min. at 60° and *p*H 4 has no effect on the serological activity, but material that is flocculated specifically by virus antiserum can be removed from such solutions by centrifugation for a few minutes at 5000 r.p.m. Heating for longer periods in these conditions increases the amount of this material, but it also causes the spontaneous separation of a precipitate. At *p*H 6 more intense heating is needed to produce this effect. Table 3

Table 3. *Effect of heating purified bushy stunt virus solutions at* p*H 6 and 80° for various times on the serological activity*

Time of heating min.	Appearance of heated fluid	Serological titre: Before centrifuging	Serological titre: After centrifuging
0	Clear	1/320,000	1/320,000
7·5	Slightly opalescent	1/320,000	1/160,000
15	Opalescent	1/160,000	1/ 40,000
30	Opalescent and slight flocculation	*1/ 80,000	1/ 5,000
60	Considerable flocculation	*1/ 10,000	No ppt. at 1/2,000

* Tests made on supernatant fluid after floccules had settled.

shows the results of heating 0·1 % solutions of virus in 0·1 *M* phosphate buffer at *p*H 6 for various times at 80°.

The effect of miscellaneous treatments. The nature of the changes in the virus particle when it is rendered non-infective by an amount of heating insufficient to cause denaturation is unknown. As treatment with H_2O_2 has a similar effect, it was thought that oxidation might be responsible. Tests were made therefore to compare the effects of heating virus solutions at *p*H 6 in air and *in vacuo*, but no differences were found. In one such test, the unheated control gave an average number of lesions per leaf at 10^{-3}, 10^{-4} and 10^{-5} of 79, 57 and 22, while those heated in air and *in vacuo* for 10 min. at 60° gave 19, 6 and 1·5 and 21, 8 and 2 respectively. Similarly, attempts to preserve the infectivity of bushy-stunt virus in expressed sap by the addition of reducing agents has failed. Indeed, the addition of 5 % Na_2SO_3 caused a rapid inactivation of a type resembling that caused by heating or ageing. Within 24 hr. at room temperature the sap had lost its infectivity, but from it we isolated a crystalline and serologically active product indistinguishable from a normal virus preparation except for its complete lack of infectivity. This type of inactivation is not reflected by any change in the sedimentation constant or in the phosphorus or carbohydrate contents. This is shown in Table 4, where sedimentation constants and analytical figures for virus preparations

Table 4. *Sedimentation constants and carbohydrate and phosphorus contents of active and non-infective purified virus preparations*

		Phosphorus %	Carbohydrate %
Fully active virus	$S_{20} = 130{\cdot}9 \times 10^{-13}$	1·47	6·0
Virus inactivated by heating at 59–60°	$S_{20} = 130{\cdot}1 \times 10^{-13}$	1·44	6·5
Virus inactivated by ageing	$S_{20} = 130{\cdot}2 \times 10^{-13}$	1·53	6·6

made non-infective by various treatments are given. The analytical methods were those used in our earlier paper. Sedimentation constants were measured by Dr A. G. Ogston and are set out in the Addendum to this paper.

The effect of freezing purified virus preparations

We have already briefly noted the inactivation of purified preparations of bushy stunt virus by freezing [Bawden & Pirie, 1938*a*]. These experiments were made only with dialysed solutions containing 5–20 g./l. and near the isoelectric point [*p*H 4·11, McFarlane & Kekwick, 1938]. Stanley [1940] found that freezing infected leaves did not destroy the virus, and we have also found that freezing plant tissues or expressed infective sap does not inactivate. We have now made experiments on the effects of freezing the virus in different conditions and find that the rate and extent of inactivation are affected by a wide range of variables. The variables we have considered are concentration of virus, *p*H, duration of freezing, temperature to which the preparation is taken, and the presence of other materials.

Unless otherwise stated, the virus preparations used in this work were purified and dialysed. So far as we know they were free from any material

other than virus. Some preparations were made by the old method, which involved heating the sap to 60°, and some by the method described in the preceding paper. Several comparisons have been made in which these two products were frozen under the same conditions and no differences have been found in their behaviour.

No experiments made at temperatures below −20° are reported in this paper. Thus, freezing means the more or less complete separation of crystalline ice, and our experiments have little in common with those [e.g. Luyet, 1937; Luyet & Thoennes, 1938] in which vitrification is induced by sudden, extreme cooling.

All the experiments on freezing were carried out in $3 \times \frac{3}{8}$ in. test tubes. The mixture of virus solution and the other components of the system was carefully pipetted in to form a pool at the bottom of the tube without wetting the walls; in general 0·1–0·4 ml. was frozen. A metal stand, previously cooled, held the tubes in a nearly horizontal position in a part of the refrigerator at the required temperature. Freezing generally occurred after a few minutes, but if it did not the mixture was seeded with about 0·005 ml. of ice carried on a cooled rod. In some experiments, especially those in which only short periods of freezing were used, the tubes were partly immersed in an ice and alcohol freezing mixture at the required temperature. This was in a glass vessel so that the moment of freezing could be easily noted. No differences have been detected between mixtures freezing at the slightly different rates that these different procedures probably involve.

After thawing, the amount of insoluble material was estimated by eye; apparently complete precipitation was called 3, substantial precipitation but a still opalescent supernatant 2, and a trace only of precipitate was called 1. The *p*H of the mixture was brought to 5·5 by the addition of *p*H 6·0 phthalate buffer or, in the experiments run at low *p*H's, phthalate to which an excess of NaOH had been added. Before testing for serological activity and infectivity the mixture was diluted with 0·9% NaCl solution so that its virus content, in terms of the initial amount of virus present, was 0·2 g./l., and centrifuged.

Influence of the concentration of virus. Table 5 shows that, at *p*H 4·1 and 2 hr. freezing, inactivation is complete only when the virus concentration exceeds 10 g./l. It also shows that the inactivated virus becomes insoluble. The results here are in good agreement with those of Nord and his collaborators in a series of papers on 'cryolysis', e.g. Nord [1933], Nord & Lang [1935], Holzapfel & Nord [1938 *a*, *b*] and Kausche & Holzapfel [1940]. They find, using a wide range of colloidal materials, that changes leading to an aggregation of the particles are favoured by an increase in the concentration of the solution that is being frozen, and that dilute solutions tend to disaggregate. An aggregation leading, as with bushy stunt virus, to the separation of a precipitate was not observed in Nord's systems, but it is a very well-known result of freezing other systems; soil [Jung, 1931] and normal leaf protein [Bawden & Pirie, 1938*c*] may be mentioned as examples, and the similar behaviour of agar is the basis of the methods used for its commercial preparation.

Table 5. *Effect of freezing purified preparations of virus at* p*H 4·1; influence of virus concentration*

Conc. of virus (g./l.)	Amount of material insoluble after thawing (arbitrary units, see p. 73)	Serological end-point
20·0	3	No ppt. at 1 : 1,000
10·0	3	1 : 1,000
5·0	3	1 : 4,000
2·5	2	1 : 32,000
1·25	1	1 : 128,000
Unfrozen control	0	1 : 320,000

The virus was dissolved in *M*/20 phthalate buffer solution at *p*H 4·1 and the samples were held frozen at −10° for 2 hr.

Influence of p*H.* Although Table 5 shows that concentration is an important factor in determining the degree of inactivation on freezing, *p*H is even more important. When solutions of the same concentration as those listed in Table 5 are frozen at *p*H 3·5, for example, even the most dilute is completely inactivated. Inactivation also occurs in solutions more dilute than these. At *p*H 5, on the other hand, inactivation is incomplete even after 12 hr. freezing. The results of an experiment in which a series of tubes containing 1 g. virus/l. were frozen at different *p*H values are set out in Table 6. It is clear from this experiment that an acid solution favours inactivation.

Table 6. *Effect of* p*H on purified virus preparations*

*p*H	Amount of material insoluble after thawing (arbitrary units, see p. 73)	Serological end-point
3·5	3	Less than 1 : 5,000
4·0	3	1 : 40,000
4·5	2	1 : 80,000
5·0	1	1 : 200,000
5·5	0	1 : 400,000
6·0	0	1 : 400,000
Unfrozen control	0	1 : 400,000

1 g./l. solutions were held frozen at −10° for 12 hr. in *M*/20 phthalate buffer.

Influence of duration of freezing. The time for which the material is held frozen can also affect the extent of inactivation. In general, the amount of inactivation increases with the duration of freezing. Clear-cut results can be obtained when the differences in duration of freezing are large, but, as perhaps might be expected, we have been unsuccessful in getting consistent results when comparing

times of freezing that do not differ greatly from one another. Table 7 illustrates these phenomena at *p*H 4·5; at other *p*H values the extent of inactivation also depends on the duration of freezing. Under the conditions of these experiments all the liquid has apparently solidified within a few seconds of the

Table 7. *Effect of duration of freezing on purified virus preparations*

Duration of freezing (min.)	Amount of material insoluble after thawing (arbitrary units, see p. 73)	Serological end-point	Av. no. of lesions per leaf at 10^{-4}	Av. no. of lesions per leaf at 10^{-5}
0	0	1 : 320,000	133	57
10	0	1 : 320,000	131	31
40	0	1 : 160,000	124	18
225	1	1 : 160,000	113	28
450	1	1 : 160,000	47	15
1465	3	1 : 40,000	5	1
1930	2	1 : 80,000	5	1
2410	3	1 : 20,000	3	1

0·1 ml. lots of 10 g./l. virus solution in *M*/30 phthalate buffer at *p*H 4·5 were frozen for the time stated.

first signs of ice formation, for there is considerable supercooling and the volumes of fluid are small, whereas the surface exposed to cold and the heat capacities of the vessel and stand are large. This apparently almost instantaneous solidification, however, does not necessarily imply immediate complete freezing. Moran [1926], by following the volume changes in a gel containing 44 % of gelatine, showed that freezing was only complete after 26 days at $-11°$, and Kistler [1936] reached a similar conclusion with emulsions of water and toluene. The latter reviews the literature of the freezing of colloids as does Blanchard [1940]. Our mixtures have a much lower colloid content than those used in the experiments of the workers referred to above, and we have no evidence that incomplete inactivation in short exposures to cold is in fact due to incomplete freezing at $-10°$. However, this is the simplest explanation of our results and may well be adopted until some evidence against it is forthcoming.

Influence of freezing to different temperatures and in the presence of various other materials. There are three obvious ways in which another solute may affect the inactivation of bushy stunt virus by freezing. First, if the eutectic temperature of the substance added is lower than the temperature to which the mass is frozen some of the solution will remain unfrozen; if enough of this solution is available to dissolve the virus, that is to say if the ratio of solute concentration to virus concentration is high enough, there is no reason why the virus should be affected by the freezing. In the second place, it may delay the establishment of complete freezing throughout the mass of the material; if a substance is acting in this way an increase in the duration of freezing should neutralize the effect. Finally, the solute may combine with the virus or precipitate it from solution; the complex or the precipitate may have a different stability from the normally dissolved virus when the surrounding water is frozen. Phthalate buffer was present in all the freezing experiments described in this section, and also in the three described above, in which it was necessary to control the *p*H. The solubility of phthalate is so low that we have no reason to think that it exerts any protective action at $-10°$ nor have any experiments suggested it. All the data on eutectic temperatures and the concentration of the eutectic are taken from Landolt and Bornstein's Tables.

Table 8 illustrates the first mechanism. Probably because of the length of time for which the samples were held frozen these results were clear cut, and the virus was either inactivated or else retained its infectivity and serological activity substantially unimpaired. Table 9 illustrates the first and second mechanisms, KNO_3 delays the inactivation somewhat, and NaCl, in spite of its low eutectic, does not, at this concentration, protect indefinitely. The amount of water that would be held unfrozen at $-10°$ by the 0·27 mg. of NaCl that is in these 0·1 ml. test samples is 0·81 mg. This amount is apparently insufficient for the permanent stability of 1 mg. of virus under these conditions. In another experiment with 10 g. virus/l. at *p*H 4·5, inactivation was not appreciable in 24 hr. at $-10°$ in the presence of 0·56 % NaCl but was nearly complete with 0·17 %. The results in a series of tubes containing the latter mixture were very variable at shorter times of freezing; this suggests that we are here at about the limit at which the amount of water held unfrozen is insufficient to preserve the virus. This hypothesis offers an explanation of the greater stability of dilute virus solutions for, other things being equal and in the presence of unavoidable traces of salt, more water is available in them for each mg. of virus. The protective action of glucose is also shown in Table 9; with as little as 0·1 % glucose there is some delay in the inactivation.

Many attempts have been made to test the protective effect of salts which have eutectic temperatures above the temperature of freezing, and which will precipitate the virus. Crystals with the usual dodecahedral form can be made by precipitating the virus at 0° with $FeSO_4$, $MgSO_4$, $ZnSO_4$, $MnSO_4$ and $Na_2S_2O_3$; the eutectic temperatures of these salts are $-1·8$, $-3·9$, $-6·5$, $-10·5$ and $-11·0°$ respectively. In some experiments these salts have been found to exert a greater protective effect than salts such as $CuSO_4$, KNO_3, KCl and $BaCl_2$ that do not precipitate the virus at 0°, but we have not been able so to define the conditions that consistent results can be obtained.

Table 8. *Effect of salts with different eutectic temperatures on purified virus preparations*

Salt	Eutectic temp.	Conc. at the eutectic of salt/100 g. of water (g.)	g. of salt/100 g. of water in the solution frozen	Amount of material insoluble after freezing at (arbitrary units, see p. 73) −10°	−20°	Activity after thawing from −10°	−20°
KNO_3	− 2·9°	12·2	4·9	3	3	−	−
KCl	−11·1°	24·0	9·6	1	.	+	.
NH_4Cl	−15·8°	22·9	9·2	0	3	+	−
NaCl	−21·2°	28·9	11·5	0	.	+	.
$MgCl_2$	−33·6°	27·5	11·0	0	0	+	+

Each test was carried out on a solution containing 40% of the eutectic concentration of the salt, 1·4 g. virus/l. and *M*/10 phthalate buffer at *p*H 4·4. In all cases the duration of freezing was 68 hr.

Table 9. *The protective effect of different materials on purified virus preparations*

In all these experiments 0·1 ml. lots of a 10 g./l. solution of virus in *M*/25 phthalate buffer at *p*H 4·0 were kept frozen at −10°. In addition the fluids contained the following substances.

Time of freezing	Salt added	Amount of material insoluble after thawing (arbitrary units, see p. 73)	Serological end-point	Av. no. of lesions per leaf at 10^{-5}	10^{-6}
1 min.	No addition	1	1:320,000	63	8
	1·6% KNO_3	0	1:320,000	29	7
	0·27% NaCl	0	1:320,000	30	12
	1·5% glucose	0	1:320,000	49	8
1 hr.	No addition	3	No ppt.	7	1
	1·6% KNO_3	2	1: 40,000	20	1
	0·27% NaCl	0	1:320,000	20	5
	1·5% glucose	0	1:320,000	23	3
24 hr.	No addition	3	No ppt.	0	0
	1·6% KNO_3	3	No ppt.	0	0
	0·27% NaCl	1	1: 80,000	3	0
	1·5% glucose	0	1:320,000	18	5
0	No addition	0	1:320,000	48	22

Effect of freezing infective sap

In infected leaves and in expressed sap, bushy stunt virus is apparently unaffected by freezing for days or even weeks. The results that have been described above give ample reason for this. Even the most highly infective sap has only a quarter of the virus concentration that is necessary for inactivation at the isoelectric point. Tomato sap has never been found with a *p*H lower than 5·6, and the salts and sugars in the sap supplement these other protective effects. After dialysis, infective sap is still not inactivated if it is frozen at its own *p*H, but it is inactivated if acidified before freezing; this is shown in Table 10. The sap from minced tomato leaves was frozen, centrifuged and dialysed for 48 hr. against frequently changed distilled water. After centrifuging again, it contained 1·8 g. solids/l. and was at *p*H 6. Samples were adjusted to *p*H 3, 4 and 5 with *N*/10 H_2SO_4, and 2 ml. lots were frozen at −10° for 14 hr. They were all centrifuged after thawing, although there was no precipitate in the one at *p*H 5, and tested. It is clear that with this dilute virus solution, inactivation took place only

Table 10. *The effect of pH on the freezing of infective sap*

*p*H of sap	Serum precipitation end-point Frozen	Unfrozen	Av. no. of lesions per leaf Frozen	Unfrozen
5	1:8	1:8	64	85
4	1:8	1:8	68	91
3	No ppt.	1:8	6	65

at *p*H 3; the serum precipitation end-point suggests that this dialysed sap contained about 0·02 g. virus/l.

Virus is also protected by the colloidal constituents of sap from inactivation by freezing. The effect is not very definite, but a solution containing 5 g. virus/l. and at *p*H 4·0 was only half inactivated in 18 hr. at −9°, when half of the final volume of solution consisted of dialysed healthy sap prepared as in the preceding experiment. When the proportion of sap colloids was only one-sixth the activity fell to one-eighth, and with less sap than this there was no protection. The colloidal material present in sap prepared in this way is largely polysaccharide,

but, since it also contains 3–4 % of N, a few experiments on protection by proteins have been made. Dialysed preparations of rabbit-serum albumin and pseudoglobulin were used and, as Table 11 shows, they had a substantial protective effect.

Table 11. *The protective effect of proteins on virus exposed to freezing*

Each mixture contained 2 g. of virus/l. dissolved in *M*/20 *p*H 3·5 phthalate buffer solution, the addition specified was also made. All except the control were kept frozen at −10° for 14 hr.

Addition	Amount of material insoluble after thawing (arbitrary units, see p. 73)	Serum precipitation end-point	Av. no. of lesions per leaf	
			10^{-4}	10^{-5}
None	3	0	0	0
1·6 % albumin	2	1 : 80,000	6	7
0·5 % albumin	3	1 : 40,000	7	0
1·6 % pseudo-globulin	1	1 : 320,000	48	11
Unfrozen control	0	1 : 320,000	169	53

Loss of infectivity without loss of serological activity

In general, loss of infectivity in frozen samples of bushy stunt virus is accompanied by a corresponding loss of serological activity and by the production of insoluble materials. However, inactivation by freezing, as by heating, is a complex process, and loss of infectivity is produced by the earliest changes. It is more difficult to make serologically active but non-infective preparations by freezing than by heating, but the results in several of our tables show that some of the frozen samples have suffered a greater loss of infectivity than of serological activity.

As with so many other aspects of this study, conditions cannot be defined which will always give a separation of the two activities on freezing. It is probable that the lack of fine control over the rate of freezing accounts for much of the variability. The most consistent results have been obtained with 0·2 % virus solutions in *M*/10 phthalate or acetate buffer at *p*H 4·25 kept at −10° for 2 hr. In about half the tests under these conditions, preparations have been non-infective although fully active serologically, and no other conditions have been found that give a higher proportion of successes. Inactivation of this type has no effect on the sedimentation constant. When such non-infective preparations were set to crystallize with $(NH_4)_2SO_4$, most of the material crystallized in the form of the usual dodecahedra, but the supernatant fluids showed a slight shimmer when shaken. Such an effect has not been noticed during the crystallization of normal preparations of bushy stunt virus, although it has been during attempts to crystallize serologically active preparations made non-infective by heating in alkaline solution. On long standing in the presence of $(NH_4)_2SO_4$ a few birefringent crystals have separated from virus preparations made non-infective, but not denatured, by freezing.

The fact that freezing can produce this type of inactivation needs to be remembered when virus preparations are being made from frozen leaves or sap, for much of the non-infective virus is indistinguishable, by the criteria we have used, from fully active virus. It is perhaps unlikely that inactivation of this type would occur under the conditions obtained in frozen sap, but if it did, infective and non-infective virus would not be separated by the ordinary purification procedure.

DISCUSSION

So many treatments can cause loss of infectivity without producing any appreciable changes in physical, chemical or serological properties, that attempts to assess the homogeneity of purified virus preparations are likely to give indecisive results until much more sensitive methods of testing for infectivity are discovered. The local lesion method is adequate for comparing the relative activity of different preparations, but it tells little or nothing about the absolute activity. The fact that the same virus preparation can give widely varying numbers of lesions on different plants of the same species, or on different leaves of the same plant, shows that the number of lesions formed in any one test bears no fixed relationship to the quantity of active virus in the inoculum. Even when using the most sensitive leaves, however, some thousands of times the amount of virus has to be applied that would be needed in theory if one virus particle can cause infection. This discrepancy may arise because of the wastage of inoculum in the relatively crude methods of inoculation used, or it may be that large numbers of active virus particles are needed to cause one lesion. The latter possibility could be tested by the method used with vaccinia [Parker, 1938; Haldane, 1939], that is to say, by determining the proportion of inoculations that fail to give any lesions when different amounts of virus are present in the inoculum. An equally probable explanation, however, is that even the best preparations of virus yet made consist largely of virus that has lost infectivity but not serological activity. Although this type of inactivation is a complicating factor in work on the purification of viruses, its occurrence makes viruses particularly suitable material for studying the earliest changes that lead to denaturation. With proteins that have no specific biological activities, changes can only be detected when the physical, chemical or serological properties have been altered measurably.

Ideally, a pure virus preparation would consist of particles all identical in size, constitution and ability

to cause infection, but there is no evidence that this ideal has ever been achieved. Deviations from it can arise in four main ways. First, the purified preparations may still contain material that is either a constituent of the normal host or a product of infection that is inessential for infectivity. The detection of such contaminants should not present serious problems, and it is probable that preparations of some viruses have been made substantially free from them. In this connexion it should be remembered that, although it may be legitimate to regard the more active of two preparations with equal solid content as the more pure, all contaminants do not necessarily reduce infectivity and serological activity [Pirie, 1940]. Secondly, even when all the particles in a virus preparation are fully active, these particles may be of different kinds. Most preparations of tobacco mosaic virus fall into this category, for even cultures derived from single local lesions are often mixtures of virus strains [Jensen, 1936]. Thirdly, during the course of isolation some or all of the virus particles may have become modified so that, although the final product consists solely of virus particles or of particles derived from them, these may vary in activity. The detection of inactive contaminants arising in this manner presents serious problems only when the modification is slight. Finally, all the virus particles in an infected leaf may not be equally infective although inseparable by the available methods. Particles less active than the ideal may be stages in the production of fully active virus or they may be particles that have suffered inactivation. The first alternative would offer a ready explanation for Spencer's [1941] observation that, weight for weight, tobacco mosaic virus from recently infected plants is less infective than virus from plants infected for longer periods. The second might also be expected as it is analogous to the type of inactivation that occurs in sap extracted from plants suffering from bushy stunt or tobacco necrosis.

In the past the thermal inactivation point has been widely used as a property for characterizing individual viruses, and with most viruses different workers have usually agreed within narrow limits about the temperature at which 10 min. heating causes loss of infectivity in expressed sap. The data on bushy stunt and tobacco necrosis viruses, however, have been conflicting. Provided the *p*H and other conditions of heating are controlled, reproducible results are readily obtained with potato virus '*X*' and tobacco mosaic virus. But with the same preparation of bushy stunt virus, the thermal inactivation point may vary over 15° in successive tests. These differences seem to arise because of the different coefficients of thermal inactivation for the different types of viruses. Loss of infectivity with virus '*X*' and tobacco mosaic virus is closely linked with denaturation and coagulation of the protein and it has a large temperature coefficient, so that the precise temperature to which the preparation is heated is of the greatest importance in determining loss of infectivity. With bushy stunt virus, on the other hand, loss of infectivity is not closely linked with denaturation and the former has a small temperature coefficient. Thus the exact temperature of heating is of little more importance in determining the apparent point of inactivation than other factors such as concentration of inoculum and susceptibility of the test plants. With such viruses, determinations of the thermal inactivation point are obviously of reduced value as a diagnostic character. Measurements of the rate of inactivation at different temperatures, however, may become of value in differentiating between those viruses with widely different coefficients of thermal inactivation. In our earlier paper on bushy stunt virus, we stressed inactivation by freezing as a property that sharply differentiated this from the other viruses we had studied, for none of the others had been affected by freezing. However, we did not then realize that the inactivation of bushy stunt virus was so dependent on the precise conditions of freezing, and other viruses may be similarly inactivated if the freezing is done under more critical conditions.

SUMMARY

Tomato bushy stunt virus loses its infectivity when heated insufficiently to cause denaturation and loss of serological activity. The temperature coefficient for loss of infectivity is small and for loss of serological activity is large. The amount of heating needed for denaturation varies with the *p*H.

No differences have been found between the chemical and physical properties of non-infective, but serologically active, material and those of fully active preparations.

The rate of inactivation by freezing is increased by increases in the concentration of the virus, in the duration of freezing and in the acidity of the fluid. The virus is protected from inactivation by salts and some other substances. The efficiency of different salts depends on the salt : ice : water eutectic temperature.

In general, loss of infectivity is accompanied by the separation of a precipitate and loss of serological activity, but in some conditions freezing destroys infectivity without altering serological activity.

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Note added 19 Nov. 1942. Since this paper was written a comprehensive review (Breedis, C. [1942], *J. exp. Med.* **76**, 221) on the effects of freezing on a variety of biological materials has appeared. It also contains experiments showing the importance of controlling the rate of freezing in the destruction of leukaemia cells.

Addendum: Examination of Bushy Stunt Virus in the Ultracentrifuge

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with the technical assistance of W. Weinstein

Nine samples of bushy stunt virus were examined in a Svedberg oil-turbine ultracentrifuge by the 'diagonal schlieren' method [Philpot, 1938]. The speed of rotation was 300–310 rev./sec.; the solutions, of concentration 0·35 or 0·25 % in 0·1 *M* NaCl with 0·01 *M* buffer (acetate *p*H 4·7 or phosphate *p*H 6·8), were contained in a 12 mm. cell.

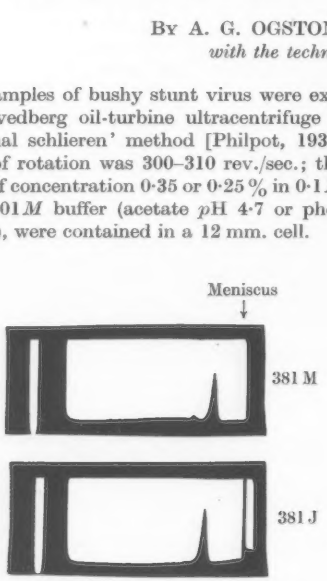

Fig. 1.

The results and details are given in Table 1 and specimen schlieren diagrams in Fig. 1.

All the preparations (except 381 M which contains a small amount of a more rapidly sedimenting component) appeared to be homogeneous. In three cases the fraction of the total refracting protein which appears in the boundary was measured by the method of Philpot [1939]; these figures are given in Table 1; but because of the sharpness of the boundaries, and the smallness of the areas measured, the figures are not very accurate; they indicate that the samples are largely homogeneous.

The sedimentation constants obtained here agree with the mean value obtained in five other laboratories on Lauffer & Stanley's [1940] virus, including the value of 133 obtained by McFarlane. It differs significantly from the value of 146 which McFarlane & Kekwick [1938] obtained on an earlier virus preparation by Bawden & Pirie [1943], but I am asked by these authors to state that this value is probably too high because of irregularities of temperature in the rotor of their Svedberg equilibrium centrifuge, which they had not suspected at the time. Recently the same virus preparation (bushy stunt 515J of Bawden & Pirie) has been examined in both laboratories (0·25 % in acetate buffer *p*H 4·0 of ionic strength 0·02) and the mean of three values (Ogston) was 129, while the mean of four values (McFarlane & Kekwick) was 133.

Table 1. *Examination of bushy stunt virus in the ultracentrifuge*

Run	Index no.	Type of product	Conc. %	*p*H	$S^{20°}_{w} \times 10^{-13}$	Fraction in boundary
270	381 J	Unheated	0·25	4·7	135·0	0·87
269	368 G	Unheated	0·25	4·7	130·3	—
274	381 K	Unheated	0·25	4·7	133·8	—
275	431 B	Heated after purification	0·25	4·7	130·1	—
273	381 M	Heated after purification	0·25	4·7	133·4	—
					225	*ca.* 0·05
266	363 G	Prep. from heated sap	0·25	4·7	130·8	0·93
267	363 G	Prep. from heated sap	0·25	6·8	135·9	—
268	350 B	Material inactivated by lying in sap	0·35	4·7	130·2	0·89
297	531 A	Inactivated by freezing	0·25	4·7	132·7	—
298	531 B	Inactivated by freezing	0·25	4·7	131·1	—

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Partition Chromatography in the Study of Protein Constituents

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In the present paper we report new applications and developments of partition chromatography* [Martin & Synge, 1941*b*] in the study of amino-acids and peptides. We have further developed the technique, aiming first at the isolation and identification of protein constituents, both amino-acids and peptides, and secondly, at a reliable micro-analytical procedure for the determination of as many different amino-acids as possible in the same sample of a complete hydrolysate of a protein or peptide.

Here we describe the more technical aspects of the work and give a detailed account of the micro-method for monoamino-acids at its present stage of development, together with the results obtained with it on wool and gelatin hydrolysates. The two following papers illustrate further the application of our new methods to particular problems in protein chemistry. Throughout the present work we have employed, as previously, the acetyl derivatives of the amino-acids and peptides.

* We employ the term 'partition chromatography' at the suggestion of Dr E. Lester Smith, to distinguish it from the classical adsorption chromatography. Our earlier term 'liquid-liquid chromatography' was liable to confusion with the fractional elution procedure sometimes called 'liquid chromatography'.

EXPERIMENTAL

Preparation of silica gel for chromatography

Adsorption by the silica of the substances undergoing analysis could seriously alter their behaviour from that to be expected were their partition between the two liquid phases the only determining factor. There must be an adequate concentration of anti-adsorbent substance in the solvent employed [Martin & Synge, 1941*b*]. Silica gel prepared in different ways has very different adsorption properties, and the more adsorbent gels may require unsuitably high concentrations of alcohol before they are useful. Adsorption by the silica may manifest itself as 'tailing' of the solutes undergoing analysis, and results in imperfect separation. This is the behaviour to be expected for adsorption of the 'Freundlich' type, where, for low concentrations, the adsorption is relatively much greater than for high ones. 'Ageing' of the freshly precipitated gel under dilute acid before drying it is essential for the production of a suitable colour with methyl orange (MO), but if ageing is continued for much longer than prescribed, a serious increase in adsorbing power may result. Deviations from the precipitation procedure given below (order of addi-

tion, concentration, temperature, etc.) result in less satisfactory products. Thorough acidification during precipitation and ageing is essential. Otherwise, low recoveries of the first acid band moving down the column will result. This is presumably due to neutralization by retained Na^+ ions, and explains the low recovery of acetylphenylalanine noted previously.

Detailed procedure. The silica gel is prepared according to Martin & Synge [1941*b*, footnote]. We give here a fuller description.

Commercial water-glass (140° Tw.-Jos. Crosfield, Ltd., Warrington) is diluted to 3 vol. with distilled water containing a little MO. 10*N* HCl is added in a thin stream with vigorous stirring, addition being interrupted at intervals and stirring continued to get efficient mixing. The solution changes first slowly and then rapidly to a thick porridge and all but the smallest lumps are broken up by stirring. When the mixture is permanently acid to thymol blue, addition of HCl is stopped and the mixture is kept 3 hr. It is filtered on a Buchner funnel and washed with distilled water (approx. 2 l./250 g. dry gel) without allowing the precipitate to crack. The gel is then suspended in *N*/5 HCl and aged 2 days at room temperature. It is again filtered and washed in the same way with distilled water (approx. 5 l./250 g. dry gel) until the washings are free from MO. Finally, the gel is crumbled and dried at 110° in an air oven. With such a preparation the addition of 53% w/w of indicator solution to the dry gel should be satisfactory with the butanol-chloroform or propanol-cyclohexane mixtures. The dry gel can be stored in a closed vessel for long periods without deterioration.

Indicators

MO has been employed throughout these experiments for indicating the presence of the acetyl derivatives. Most of the usual indicators of suitable *p*H range which we have studied are very readily extracted from the aqueous phase by the developing solvent. MO is not free from this defect, and is slowly removed by the solvent, particularly if the alcohol content of the latter is high. When this happens, the column becomes colourless from above downwards, and is useless for the visual separation of solutes whose bands move much slower than that of acetylglycine.

The anthocyanin series promises to yield suitable indicators. Of those tested, an aqueous solution (0·05%) of pelargonin chloride (prepared from petals of the scarlet dahlia 'Coltness Gem' [cf. Willstätter & Mallison, 1915]) has proved useful. It is not readily 'leached' by solvent mixtures quite rich in alcohols; its sensitivity to acid is not (like that of MO) markedly depressed by alcohols; and it is free from N and S.

The presence of MO in the fractions obtained from the chromatograms has not interfered with the titration of the acetyl derivatives described below, but the indicator has sometimes been difficult to eliminate in the course of preparative work.

The 'background' colour of the MO in the chromatograms is greatly affected by the nature of the developing solvent, as well as by the silica, and may vary from bright pink to bright yellow. The solvents whose use we describe below all give usable orange background colours, although 30% propanol-cyclohexane gives a rather yellow and insensitive column. Good lighting is essential for making the best 'cut' when the bands are not widely separated. In the absence of bright daylight we have used a 80W B.T.H. fluorescent lamp with success.

Behaviour of amino-acid and peptide derivatives in different solvent systems

We have studied in detail three different solvent systems in which the acetyl derivatives of the mono-amino-acids and some of their peptides have suitable distributions, viz.: (1) chloroform-*n*-butanol-water, (2) cyclohexane-*n*-propanol-water, (3) ethyl acetate-water. Tables 1 and 2 give the band-rates of a number of those compounds in chromatograms employing these solvent systems. *R* has the same significance as before [Martin & Synge, 1941*b*], and may be related to the partition coefficient by the same formula [*loc. cit.*]. Since *R* depends on a number of variable factors, the values which we give must be regarded as a rough guide only. The ratios between these values for different compounds in the same column are, however, relatively constant.

Here and subsequently such an expression as 1% butanol-chloroform refers to a solution in chloroform of butanol, 1% v/v, subsequently saturated with water.

(1) *Acetamino-acids.* While in general the acetamino-acids with longer and less polar 'side-chains' move fastest on the chromatograms, there are some striking differences in their sequence in the different solvents. Thus while acetylphenylalanine travels faster than acetylleucine in chloroform-butanol-water, it is not separable from it in cyclohexane-propanol-water. Acetyltryptophan, acetylmethionine and acetylproline are also relatively retarded in the latter solvent. The same is generally true for this 'more polar' class of acetamino-acids in ethyl acetate-water as against chloroform-butanol-water. In ethyl acetate-water, however, *N*-acetyltyrosine is considerably accelerated.

In general, the less polar solvent (cyclohexane) discriminates more between these classes of compound than does the more polar solvent ($CHCl_3$). However, a single parameter such as 'polarity' probably cannot be employed for giving a complete

Table 1. *Band rates of acetamino-acids with various solvents*

(Figures are values of R)

Developing solvents	Butanol-chloroform 1%	Butanol-chloroform 3%	Butanol-chloroform 17%	Propanol-cyclohexane 5%	Propanol-cyclohexane 30%	Ethyl acetate
Acetylphenylalanine	0·5	0·9	Fast	0·3	Fast	Fast
Acetyl*nor*leucine	0·4	—	Fast	—	—	—
Acetylleucine / Acetyl*iso*leucine	0·3	0·6	Fast	0·3	Fast	Fast
Acetyltryptophan	0·3	0·6	Fast	0·15	Fast	—
Acetylvaline / Acetyl*nor*valine	0·15	0·3	Fast	0·15	Fast	0·9
Acetylmethionine	0·15	0·3	Fast	0·09	0·7	0·8
Acetylproline	0·15	0·3	Fast	0·04	0·3	0·2
α-Acetaminobutyric acid	0·07	—	—	—	—	0·5
Acetylalanine	0·025	0·04	0·35	0·04	0·3	0·2
N-Acetyltyrosine	0·02	0·04	0·7	—	0·6	Fast
Acetylglycine	Slow	Slow	0·15	Slow	0·15	0·1
Acetylaspartic acid / Acetylglutamic acid / *NN'*-Diacetylcystine / *NN'*-Diacetyllysine	Slow	Slow	0·1–0·2	—	—	—
N-Acetylhydroxyproline	Slow	Slow	0·07	Slow	0·1	0·06

theoretical description of such systems. Our experience suggests that new and useful solvent mixtures having specific effects with particular side-chains remain to be found.

We have not yet effected a satisfactory separation of leucine from *iso*leucine, or of valine from *nor*valine, and are now unable to confirm the separation of the former using 0·5% butanol-chloroform previously reported [Martin & Synge, 1941*b*]. We have not observed any separation of an optically active substance from the corresponding racemic compound in the course of this work.

(2) *Acetylpeptides.* The evidence presented in Table 2 suggests that acetylpeptides travel in general at rates intermediate between those of the acetyl

Table 2. *Band rates of acetyldipeptides with various solvents*

(Figures are values of R)

Developing solvents	Butanol-chloroform 1%	Butanol-chloroform 17%	Propanol-cyclohexane 5%	Propanol-cyclohexane 30%	Ethyl acetate
Acetylleucyl-leucine	1·0	Fast	—	—	Fast
Acetylleucyl-glycine / Acetylglycyl-leucine	0·04	0·6	0·04	1·0	0·5
Acetylglycyl-glycine	Slow	0·04	—	—	0·06

derivatives of their constituent amino-acids, and this is confirmed by experiments on the products of the partial hydrolysis of gelatin [Gordon, Martin & Synge, 1943*b*].

The data of Tiselius [1942] on the behaviour of free amino-acids and peptides in adsorption chromatograms packed with carbon and developed with aqueous solutions show a considerable difference in sequence from that obtained with our systems. Use of the two methods in succession might lead to highly effective separations of peptides. However, before Tiselius's chromatograms can be used for the isolation of substances it will be necessary to eliminate 'tailing' of the bands, which the adsorption data given by Cheldelin & Williams [1942] lead us to expect [cf. Wachtel & Cassidy, 1942]. Tiselius's use of 'moving boundary' observation methods with a chromatogram continuously fed with the mixture to be investigated is not interfered with by 'tailing'—which, indeed, increases the sharpness of the front boundary of each solute. The disadvantages of this procedure for isolative work are manifest, only a small portion of the least adsorbed component being readily obtainable in a state of purity.

Preparation and properties of acetyl derivatives studied

The compounds referred to in Tables 1 and 2 were described by Synge [1939] with bibliography, except for the following. Compounds reported here for the first time are indicated by an asterisk. All M.P.'s are given uncorrected unless otherwise stated. N determinations were by the Kjeldahl procedure. C and H determinations were carried out for us by Dr G. Weiler, Oxford.

(1) *Acetyl-*l*-valine.* The earlier preparation made by acetylation of *l*(+)-valine ('*d*-valin' of Hofmann La Roche) proved on chromatographic investigation to be a mixture containing at least 15% of impurity, probably an acetylleucine isomer. This would account for the deviations of the analytical values from the theoretical recorded by Synge [1939].

Our present material was prepared by resolution of acetyl-*dl*-valine [cf. Synge, 1939] obtained by acetylation of a specimen of *dl*-valine which we had prepared by a malonic ester synthesis from *iso*propyl bromide [Marvel & du Vigneaud, 1931; Marvel, 1940]. The acetyl-*dl*-valine was resolved directly as its brucine salt. A number of intermediate stages that would have been necessary had we employed Fischer's [1906] resolution were thus avoided.

3 g. of acetyl-*dl*-valine and 7·5 g. of brucine were dissoved in a minimum of ethanol, and ether was added to turbidity. Slow crystallization resulted, which in later preparations could be accelerated by seeding and stirring. The crystalline brucine salt was filtered off, washed with ether, and recrystallized in the same way from ethanol-ether. The recrystallized product weighed 4·2 g. Brucine was removed from the salt by treatment with NaOH in aqueous solution at 0°. After filtration of the precipitated brucine, the solution was extracted with $CHCl_3$ to remove the part remaining in solution. Dilute H_2SO_4 equivalent to the NaOH employed was then added, the mixture was evaporated to dryness *in vacuo*, and the solid residue was thoroughly extracted with boiling ethanol. On evaporation of the combined alcoholic extracts, crystalline acetyl-*l*-valine resulted, which was recrystallized from water until its optical rotation and M.P. were constant. Yield 0·9 g., having M.P. 164°, $(\alpha)_D^{19°}$ +4·0° (ethanol, $c=2$). The preparation was chromatographically homogeneous. These physical data supersede those given for acetyl-*l*-valine by Synge [1939].

(2)* *Acetyl*-dl-nor*valine*. This was prepared from a specimen of *dl-nor*valine (manufactured by Fränkel and Landau, and kindly given to us by Prof. H. A. Krebs) in the way described by Synge [1939]. The product on recrystallization from water had constant M.P. 111–112·5°. It proved, however, on chromatographic investigation to contain an impurity (at least 15%), probably an acetylleucine isomer. This suggests the occurrence of mixed crystals. As the preparation was required for qualitative chromatographic study, the presence of this impurity was not an obstacle, and we have not yet purified the compound. The elementary composition of the preparation is in agreement with that expected from the chromatographic data. (Found: C, 53·5; H, 8·27; N, 8·67%. $C_7H_{13}O_3N$ requires: C, 52·8; H, 8·17; N, 8·8%. $C_8H_{15}O_3N$ requires: C, 55·5; H, 8·67; N, 8·1%.)

(3)* *NN′-Diacetyl*-dl-*lysine* [cf. Synge, 1939; Kolb & Toennies, 1942]. Synthetic *dl*-lysine was acetylated in aqueous solution with NaOH and acetic anhydride as usual. After acidification with H_2SO_4, the aqueous solution was extracted repeatedly with ethyl acetate. The syrupy residue from evaporation of the combined extracts crystallized after keeping for several weeks. The product was readily recrystallized from ethyl acetate, in which it is rather insoluble. M.P. 140–141°. (Found: C, 52·0; H, 7·62; N, 12·1%; acid equiv. wt. 232. $C_{10}H_{18}O_4N_2$ requires: C, 52·2; H, 7·82; N, 12·2%; acid equiv. wt. 230.)

(4)* *Acetyl*-l-*aspartic acid*. This was prepared from *l*-aspartic acid by acetylation and extraction from acid solution with ethyl acetate as for the previous compound. The product was syrupy.

(5) *Acetyl*-l-*tryptophan*. This was prepared according to du Vigneaud & Sealock [1932] from *l*-tryptophan. Our product had M.P. 185–6°, $(\alpha)_D^{21·5°}$ +27·6° (water containing 1 equiv. of NaOH, $c=1$), $(\alpha)_D^{19°}$ +26·0° (ethanol, $c=1·1$). Du Vigneaud & Sealock record M.P. 189–190° (corr.), $(\alpha)_D^{31°}$ +29° (water containing 1 equiv. of NaOH, $c=1$). From $CHCl_3$ the compound was found to crystallize with 1 mol. of solvent. This was lost only slowly in a vacuum desiccator at room temperature, but was readily removed by drying *in vacuo* at 100°. Under these conditions the specimen lost 30·2% of its weight. (Loss of wt. calc. for $C_{13}H_{14}O_3N_2.CHCl_3$ is 32·7%.)

(6) *Acetylglycyl*-dl-*leucine*. Prepared according to Steiger [1934] from glycyl-*dl*-leucine [cf. Fischer & Warburg, 1905]. M.P. 176–177°. Steiger records 178–180° (corr.). (Acid equiv. wt.: Found, 223. Calc. for $C_{10}H_{18}O_4N_2$, 230.)

(7) *Acetyl*-dl-*leucylglycine*. Prepared according to Steiger [1934] from *dl*-leucylglycine [cf. Fischer & Brunner, 1905]. When crystallized from acetone containing 5% water the compound crystallized with $\frac{2}{3}$ mol. of acetone, which could be removed by drying over P_2O_5 *in vacuo* at 100°. The undried compound had acid equiv. wt. 270, and lost 14·4% of its weight on drying. (Calc. for $C_{10}H_{18}O_4N_2$, $\frac{2}{3}$ (C_3H_6O): acid equiv. wt. 268·6; loss of weight on drying, 14·3%.) The dried material regained the lost weight in an atmosphere saturated with acetone vapour, but not in an atmosphere saturated with water vapour. The dried material had M.P. 178°. Steiger records M.P. 182–183·5° (corr.). He does not mention the occurrence of acetone of crystallization.

(8) *Acetylglycylglycine*. Prepared from glycylglycine [cf. Fischer & Fourneau, 1901] by Steiger's [1934] acetylation procedure. Recrystallized from ethanol, the product had M.P. 178–179°. Fischer & Otto [1903] record M.P. 184–186°. (Acid equiv. wt.: Found, 175. Calc. for $C_6H_{10}O_4N_2$, 174.)

(9)* *Acetyl*-dl-*leucyl*-dl-*leucine*. This was prepared from the racemic leucylleucine [cf. Fischer, 1904] subsequently named leucylleucine 'A' by Fischer & Koelker [1907] and shown by them to be the racemic compound involving *d*-leucyl-*l*-leucine as opposed to *d*-leucyl-*d*-leucine. We acetylated the dipeptide with acetic anhydride and excess NaOH in aqueous solution as usual, and on acidification of the reaction mixture the acetyl compound crystallized out in 71% yield. Recrystallization from ethyl acetate yielded a product having M.P. 200°. (Found: C, 58·6; H, 9·02; N, 9·4%; acid equiv. wt. 278. $C_{14}H_{26}O_4N_2$ requires: C, 58·7; H, 9·09; N, 9·8%; acid equiv. wt. 286.)

Micro-determination of monoamino-acids

The method of analysis to be described is a development of that given by Martin & Synge [1941*b*], and is based on the observations reported above. By the use of additional solvent mixtures it is now possible to determine the following amino-acids and groups of amino-acids in a protein hydrolysate, viz. phenylalanine, leucine + *iso*leucine, valine (+ *nor*valine, if present), methionine, proline, alanine, and tyrosine. Tryptophan may also be determined semi-quantitatively if it survives the treatment employed for hydrolysing the specimen [cf. Gordon *et al.* 1943*a*]. Also in certain special cases [cf. Gordon *et al.* 1943 *a*, *b*] glycine has been determined semi-quantitatively by this procedure. A satisfactory determination of lysine as its diacetyl derivative could be developed on these lines for use with basic amino-acid fractions prepared by electrodialysis or

precipitation with phosphotungstic acid. The need for a direct determination of this amino-acid has long been felt.

This extension of the range of the method is due to two novel features:

(1) After the faster moving constituents of the mixture undergoing analysis have left the column, some of the remaining slow-moving constituents are accelerated and run out by the use of a higher percentage of alcohol in the developing solvent. This has brought alanine, tyrosine, etc., within the scope of the method.

(2) By subjecting each fraction obtained from the butanol-chloroform chromatogram to further fractionation on a propanol-cyclohexane chromatogram, it is possible first to separate the (proline + valine + methionine) fraction into its three constituents, and secondly to remove a number of impurities (probably decomposition products resulting from prolonged treatment of the mixture with hot acid) which affect significantly the accuracy of analyses by the procedure originally described.

It is sometimes advantageous to change the order in which the chromatograms are used [cf. Gordon *et al.* 1943*a*], or to adopt different solvent mixtures. The present scheme, however, is likely to be a useful and routine approach where little is known about the constituents of the mixture for analysis. With the method described here, the amino-acids in question may in the first instance be determined on the hydrolysate of a single sample of protein with an accuracy of $\pm 5\%$. It may then be possible to devise control experiments [cf. Gordon *et al.* 1943*a*] for increasing the accuracy of the determination.

These analytical results are not affected by the degree of racemization of the amino-acids. As pointed out by Moore, Stein & Bergmann [1942], this is an inherent defect of their 'solubility product' techniques.

Our new procedure yields results of accuracy comparable with that of the better routine amino-acid determinations in current use, and furthermore covers a group of amino-acids for which analytical methods have in the past been inadequate. Since, in addition, it is on the microchemical scale (about seven different components being determinable on a 25 mg. sample of protein), it may help to satisfy a long-felt need.

Preparation of material for chromatography. Further work on the acetylation and extraction procedure described by Martin & Synge [1941*b*] has led us to introduce some improvements and simplifications. The method at present in use is as follows. The HCl hydrolysate to be acetylated (corresponding to about 25 mg. of protein) is evaporated *in vacuo* to dryness in a 100 ml. Erlenmayer flask. 2 ml. of 2*N* NaOH are added and then 0·4 ml. of 6*N* NaOH. The solution is then cooled to 0°, and 0·2 ml. of acetic anhydride is immediately introduced, after which the flask is shaken and again cooled. Four further additions of 2 ml. of 2*N* NaOH, followed by 0·2 ml. of acetic anhydride, are made at 2 min. intervals and are accompanied by cooling and shaking. Thymolphthalein (as an external indicator) is used to ensure that the solution remains alkaline throughout. The solution is then allowed to stand for 10 min., care being taken to see that it remains alkaline. If this is not done anomalous bands appear which are probably due to *ON*-diacetyl hydroxyamino-acids which are formed if insufficient NaOH is present [cf. du Vigneaud & Meyer, 1932]. The *O*-acetyl groups are readily saponified by alkali in the cold. At the end of the 10 min. period, the solution is made acid (red) to thymol blue by the addition of 10*N* H_2SO_4. Next it is transferred to a 60 ml. separating funnel and extracted with five successive 50 ml. portions of 17% butanol-chloroform. The extracting solvent is used to rinse out the flask after transferring the mixture to the separating funnel. The $CHCl_3$ extracts are filtered through a dry filter paper (Whatman no. 2) into a Claisen flask from which the solvent is subsequently evaporated *in vacuo*, the temperature being allowed to rise to 100°. Finally, the extracted acetamino-acids are transferred in ethanol to a small flask, the ethanol is removed *in vacuo*, and the residue kept overnight in a vacuum desiccator over soda-lime and H_2SO_4.

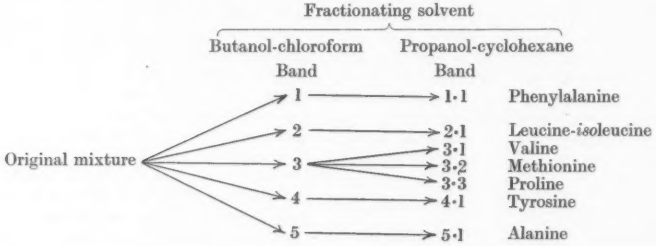
Chromatographic procedure. 3 g. of silica gel (prepared as described above) are saturated with methyl orange solution and suspended in 3% butanol-chloroform. This is introduced into a chromatogram tube having an internal diameter of 1 cm. [Martin & Synge, 1941*b*]. When the column is ready, as much as possible of the acetylated mixture is transferred to it by repeated (hot) extraction with small portions (1 ml. or less) of the solvent mixture. Each portion is allowed to drain into the gel before the next is added. After about five successive extractions, the flask containing the unextracted material is put aside for the second stage of the analysis, and the chromatogram tube is filled with the solvent mixture for development (3% butanol-chloroform).

The bands corresponding to phenylalanine (1), (leucine + *iso*leucine) (2), and (proline + valine + methionine) (3), are allowed to run out and are collected separately in pyrex flasks. The surface of the solvent is then allowed to sink until it is level with the top of the silica gel. At this point the acetylated material remaining in the flask, previously dissolved by boiling with about 1 ml. of 17% butanol-chloroform, is run on to the column. This is allowed to sink in, and after the flask has been washed out three times as before, development of the chromatogram is continued with the new solvent. This causes the bands corresponding to tyrosine and alanine, which have up to now been moving at nearly the same rate (ahead of the glycine, cystine, lysine, glutamic acid, etc.), to move faster, and at the same time draw apart from one another, the tyrosine band (4) running approximately twice as fast as the alanine band (5). These fractions are allowed to run out, and are collected separately.

Fractions 1–5 are freed from butanol-chloroform by evaporation on the water-bath *in vacuo*. Frac-

tions 1 and 2 are further analysed exactly as before, on columns containing 2 g. of silica, but using 5% propanol-cyclohexane instead of butanol-chloroform. The main band from each chromatogram is run out and collected (1·1 and 2·1, Table 3) free from any minor contaminants moving at different rates. Fraction 3 is similarly fractionated with the same solvent mixture on a rather longer column employing 3 g. of silica. Here the first band to be collected corresponds to valine (3·1). When this has run out, the solvent mixture is changed to 30% propanol-cyclohexane, and the bands corresponding to methionine (3·2) and proline (3·3) are collected separately. Fractions 4 and 5 are fractionated on columns containing 2 g. of silica and developed with 30% propanol-cyclohexane. These result in the main fractions 4·1 and 5·1. Table 3 illustrates this fractionation.

Table 3. *Scheme of fractionation*



Titration of resulting fractions. The seven final fractions are freed from solvent by evaporation *in vacuo* on the water-bath; the residue in each flask is dissolved in 3 ml. of water (by heating if necessary) and titrated against $N/100$ $Ba(OH)_2$ using bromthymol blue as internal indicator. This is used in preference to phenolphthalein because the indicator blank is smaller and it is not affected by room concentration of CO_2; further, it gives with *N*-acetyltyrosine accurate titrations of the —COOH group only. With phenolphthalein the phenolic group of this compound titrates partially, giving a high result with an unsatisfactory end-point.

For all the amino-acids determined at present except tryptophan, 1 ml. of $N/100$ $Ba(OH)_2$ corresponds to 0·14 mg. of N; for tryptophan it corresponds to 0·28 mg. of N.

Control experiments on procedure. The mixture of amino-acids and their acetyl derivatives shown in Table 4, which simulates in amino-acid composition approximately 25 mg. of wool, was refluxed in an excess of 6*N* HCl for 24 hr., and was then analysed as above.

In this experiment 3% butanol-chloroform was used for the initial fractionation because it causes more rapid movement of the bands, even though it gives rather poor separations between bands 1, 2 and 3. Thus the low recovery of phenylalanine and the excess of leucine no doubt result from the collection of some of the former material with the leucine band. Similarly, some of the leucine was collected in the proline-valine-methionine band, but this does not affect the accuracy of the analysis because, although inseparable from phenylalanine, leucine can easily be separated from proline, valine and methionine on the propanol-cyclohexane column. As previously reported [Martin & Synge, 1941*b*], good separations of bands 1, 2 and 3 can be obtained by using 1% butanol-chloroform, and unless a longer column is used, this solvent is recommended for development until bands 1 and 2 have been run out of the column.

Table 4. *Control analysis on an amino-acid mixture*

	Taken (mg. N)	Found (mg. N)	Recovery (%)
Acetyl-*dl*-phenylalanine	0·0954	0·088	92
Acetyl-*d*-leucine	0·2420	0·310	104
Acetyl-*d*-*iso*leucine	0·0566		
Acetyl-*dl*-valine	0·1637	0·156	95
l-Methionine	0·0226	0·021	93
Acetyl-*l*-proline hydrate	0·1799	0·189	105
Acetyl-*dl*-alanine	0·1700	0·165	97
l-Tyrosine	0·0795	0·082	103
Acetyl-*l*-tryptophan	0·0376	—	—
Glycine	0·5330	—	—
l-Cystine	0·3455	—	—
l-Glutamic acid	0·2555	—	—
l-Aspartic acid	0·1295	—	—
dl-Serine	0·2478	—	—
dl-Threonine	0·1928	—	—
l-Arginine monohydrochloride	0·9280	—	—
dl-Lysine dihydrochloride	0·1330	—	—
l-Histidine monohydrochloride	0·0417	—	—
Ammonium sulphate	0·3415	—	—
Total	4·1956		

No tryptophan survived the conditions to which the mixture was subjected—had any done so it would have been detected during the further fractionation of fraction 2 with propanol-cyclohexane. The work reported in our paper on gramicidin

[Gordon *et al.* 1943*a*] shows that substantial recoveries of acetyltryptophan result when tryptophan is present, with simpler amino-acids, in large amount, and when the hydrolysis is conducted with exclusion of air. Even here, however, some destruction of tryptophan occurs.

In the control experiment here reported, the following 'artefact bands' were noted during refractionation of the materials from the first column with propanol-cyclohexane. All were present in small amount.

Fraction 1. (*a*) A band travelling twice as fast as the phenylalanine band. (*b*) A band remaining near the top of the chromatogram.

Fraction 3. (*a*) A band travelling between the methionine and proline bands. (*b*) A band travelling just ahead of the proline band, but so close that its adequate elimination proved difficult.

Fraction 4. (*a*) A band moving at about half the rate of the tyrosine band.

It seems possible from the high value of the tyrosine recovery, that a contaminant not separable from it by this procedure is present. Although none of these artefact contaminants is present in very large amount, they render quite inaccurate the single fractionation procedure originally given by Martin & Synge [1941*b*]. Moreover, as they are probably formed by alteration of the original components of the mixture, this may explain the lower accuracy of the present determinations where a known complicated mixture was heated with acid in the open, as compared with those reported by Gordon *et al.* [1943*b*] for a much simpler known mixture heated *in vacuo*. These considerations are important and introduce a factor of uncertainty, stressed by Moore *et al.* [1942], into the process of relating the results of analysis of a protein hydrolysate to the amino-acid composition of the intact protein.

Analysis of wool and gelatin

Wool (Merino 64's) and gelatin (from the same batch of cow-hide gelatin studied by Gordon *et al.* 1943*b*) were subjected to analysis by the procedure given above. The results of the analysis (uncorrected) are shown in Table 5, together with previous data on wool. The analysis of gelatin is correlated with the data of other workers in an accompanying paper [Gordon *et al.* 1943*b*].

Before acetylation the wool hydrolysate had been extracted with $CHCl_3$ to remove non-amino-acid constituents [cf. Martin & Synge, 1941*a*]. The artefact contaminants noted in the control analysis were all present also in the wool hydrolysate, but one additional artefact was noted, which appeared as a slow-moving band in fraction 2.

In the gelatin hydrolysate, contaminants 1(*a*), 3(*a*), 3(*b*) and 4(*a*) were absent, but two faint bands (one ahead of and one behind the leucine band) were noted during the fractionation of band 2. A faint band running at about half the rate of the proline band was noted in the fractionation of band 3. Some of these differences may be attributed to the absence of tryptophan from gelatin; the absence of cystine is probably also of significance.

The most striking feature of the analysis of wool is the phenylalanine value, which has been much lowered by the elimination of 'artefacts'. The possibility that aromatic constituents as yet unknown are present is suggested by Block's [1939] higher value, obtained by the not very specific colorimetric procedure.

Otherwise the results are in substantial agreement with those previously recorded [cf. Martin & Synge, 1941*a*, for bibliography]. The value for alanine is the first to be reported for wool since Abderhalden & Voitinovici's [1907] ester analysis, and is in substantial agreement with their figure.

SUMMARY

1. Technical and theoretical aspects of partition chromatography are discussed in relation to the separation of amino-acids and peptides in the form of their acetyl derivatives.

2. New data are presented on the behaviour of a number of these compounds in different solvent systems.

3. A micro-analytical procedure is described for the simultaneous determination of phenylalanine,

Table 5. *Analyses of wool and gelatin*

(N as % of total N)

	Gelatin	*Wool*			
	Present detn.	Present detn.	Martin & Synge [1941*b*]	Other detns.	
Phenylalanine	0·4	0·8	1·9	1·9	
Leucine-*iso*leucine	3·9	6·2	7·4	7·2	
Valine	1·7	3·9		3·4	Martin & Synge [1941*a*]
Methionine	0·3	0·2	8·2	0·4	Martin & Synge [1941*a*]
Proline	9·6	5·2		4·9	Martin & Synge [1941*a*]
Tyrosine	? Trace	2·8	—	2·1	Marston [1928]
				2·3	Block [1939]
Alanine	8·3	3·9	—	4·1	Abderhalden & Voitinovici [1907]

leucine isomers, valine, methionine, proline, alanine and tyrosine in the hydrolysate of a single sample of protein.

4. Determinations of these constituents of wool and cow-hide gelatin are reported.

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The Amino-Acid Composition of Gramicidin

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The bactericidal substance gramicidin, isolated by Dubos *et al.* from cultures of various bacteria [for bibliography cf. Dubos & Hotchkiss, 1942] is of polypeptide character, yielding on acid hydrolysis a mixture of amino-acids, some of which possess the 'unnatural' *d*-configuration [Hotchkiss, 1941; Lipmann, Hotchkiss & Dubos, 1941; Christensen, Edwards & Piersma, 1941].

We have subjected acid hydrolysates of gramicidin to analysis by our new techniques [Gordon, Martin & Synge, 1943*a*] and have been able to confirm and extend most of the data of these authors.

Taken in conjunction with the analytical results of Hotchkiss [1941], our analyses suggest that the gramicidin molecule contains 24 amino-acid residues or some integral multiple thereof. These are made up as shown in Table 1.

The presence of leucine and tryptophan residues in gramicidin has already been established by isolation and characterization [Hotchkiss, 1941; Christensen *et al.* 1941]. Christensen *et al.* also claim to have isolated alanine through its dioxpyridate and phosphotungstate, although no experimental details are given. They obtained a negative result for glycine, using the colorimetric method of Patton [1934]. We have established the occurrence in the hydrolysate of alanine, valine and glycine by isolation and characterization as the acetyl derivatives, and have confirmed in the same way the presence of leucine and tryptophan.

Table 1. *Suggested composition of minimum molecule of gramicidin*

	No. of residues	No. of atoms N
Leucine	6	6
Tryptophan	6	12
Valine	5	5
Alanine	3	3
Glycine	2	2
Hydroxyamino compound	2	2
Total	24	30

Our technique was not suitable for the isolation of the hydroxyamino compound [Hotchkiss, 1941]. We have, however, established by control experiments (see Experimental Section) that serine is

absent from gramicidin hydrolysates. Our methods would probably also reveal higher aliphatic hydroxyamino-acids, and on the evidence presented by Hotchkiss, *iso*serine would seem to be the substance whose properties agree best with what is known at present of this constituent of gramicidin.

Although our isolative procedure leads to slight racemization [cf. Martin & Synge, 1941], we have been able to confirm that the leucine of the hydrolysate is largely of the *d*-configuration, while the tryptophan has the *l*-configuration. The alanine is also largely in the *l*-form. We find, however, that the valine isolated is racemic, and since this method of isolation enabled us to prepare from wool [Martin & Synge, 1941] and from gelatin [Gordon *et al.* 1943*c*] acetylvaline retaining a large part of its optical activity, we conclude either that the valine residues of gramicidin are very readily racemized during hydrolysis, or that both *d*- and *l*-valine residues co-exist in the gramicidin molecule. It is impossible at present to choose between these alternatives. We have previously noted a similar phenomenon, viz. the occurrence of racemic phenylalanine in wool hydrolysates, and the possibility that selective racemization occurs during acid hydrolysis has been discussed in that connexion [Martin & Synge, 1941] (cf. Gordon *et al.* [1943*a*] for isolation of optically active acetylphenylalanine by a similar procedure).

Since glycine does not react with the *d*-amino-acid oxidase [Krebs, 1935], the α-amino-acids oxidized by the latter [Lipmann *et al.* 1941] must consist largely of leucine and of half the valine residues. These amount to 38·6 % of the total α-amino-acid residues in the postulated molecule, in fair agreement with the figure 43–46 % determined by Lipmann *et al.*

In Table 2 we correlate our experimental data with those of Hotchkiss [1941] and with the values calculated for the postulated minimum molecule containing 30 atoms of nitrogen.

Table 2. *Constituents of gramicidin hydrolysate*

(N as % of total N)

	Present detn.	Hotchkiss [1941]	Calc.
Leucine	20·2	16·1*	20·0
Tryptophan	40–45	37·3	40·0
Valine	16·6	—	16·7
Alanine	10·1	(25–35)	10·0
Glycine	5·3–6·6	—	6·7
Hydroxyamino N + NH_3-N	—	6·0	6·7
—OH groups (before hydrolysis) (equivs. as N)	—	7·3	6·7
α-Amino N (ninhydrin)	—	72·6	73·3
Amino-N (Van Slyke)	—	80·4	80·0
Basic groups by titration (equivs. as N)	—	80·5	80·0
Acidic groups by titration (equivs. as N)	—	84·7	80·0

* By isolation.

Our values for tryptophan and glycine are semi-quantitative only, for reasons discussed in the Experimental Section.

The method of Kendall & Friedemann [1930] used by Hotchkiss for determining alanine gives seriously high values in the presence of valine and leucine.

We have added Hotchkiss's figure for NH_3 in the hydrolysate to his determined hydroxyamino N, since the hydroxyamino compound seems a probable source of the NH_3 liberated during treatment with hot acid [cf. Hotchkiss, 1941; Gordon, Martin & Synge, 1941].

There is reasonable agreement between the experimental data and the figures calculated for the molecule postulated in Table 1.

This minimum molecule deduced by stoichiometric reasoning has the molecular weight 2790, assuming that (i) the unknown hydroxyamino compound is *iso*serine, and (ii) the gramicidin molecule is a cyclopeptide of 24 residues, formed from the free amino-acids by elimination of 24 molecules of water.

This value for the molecular weight of gramicidin is in fair agreement with that found by Tishler, Stokes, Trenner & Conn [1941] by isothermal distillation in methanol, namely, 3100, and also with the value 2500–4500 found by the same authors by diffusion measurements in *n*-butanol. The cryoscopic determinations of these authors and of Hotchkiss [1941] gave lower values. The cryoscopic data of Tishler *et al.* for gramicidin in cyclohexanol showed apparent lowering of the molecular weight at low gramicidin concentrations. If this were due to a reversible dissociation of 'chemically bound' water from the gramicidin molecule, i.e. to water which is not removable by the usual drying procedures, but which is capable of equilibrium dissociation under conditions where it could be substituted by molecules of solvent, the results would be readily explicable. Such dissociated water, while affecting the cryoscopic data, would distil with the methanol in the isothermal distillation experiment, and thus escape detection. (Tishler *et al.* [1941] do not describe their drying procedure in detail.) If this were so the cryoscopic behaviour of gramicidin in cyclohexanol would be profoundly modified by drying it 'azeotropically' in dilute solution in cyclohexanol or similar solvent. Tishler *et al.* [1941] mention that gramicidin is hygroscopic. Abderhalden & Gohdes [1931] have described the binding of water by alanine peptides.

In the absence of further data it seems reasonable to conclude that the minimum molecular weight deduced for gramicidin by stoichiometric reasoning is the true molecular weight, and that we are dealing with a 24-cyclopeptide.

The equivalent weight given by Tishler *et al.* [1941] for gramicidin in combination with flavianic

acid (2986, 3036 ± 300) is in substantial agreement with the molecule postulated.

Assuming that the molecule contains two residues of *iso*serine and no chemically bound water, and that it is a cyclopeptide without terminal —NH_2 or —COOH groups, the molecular formula can be deduced, namely, $C_{146}H_{202}O_{26}N_{30}$, giving a calculated elementary composition of C, 62·8; H, 7·25; N, 15·05%. Hotchkiss & Dubos [1941] record C, 62·7; H, 7·59; N, 14·8%. Tishler *et al.* [1941] agree about the C and N content, but find H, 7·36–7·41%.

In the absence of information on the exact nature of the hydroxyamino compound and in view of possible 'chemically bound' water, one can only say that the composition postulated for gramicidin is not incompatible with its determined elementary composition.

The stoichiometric data obtained in the present study are in striking agreement with the Bergmann-Niemann hypothesis on protein structure, in that the total number of residues in the molecule, and the number of each species of residue except valine, are expressible in the form $2^m \times 3^n$, where m and n are integers. Even with the valine residues, in view of their unascertained optical character, it would be possible to postulate three *d*-residues and two *l*-residues or *vice versa* as independent components. Alternatively, gramicidin may not be a homogeneous substance but a 'molecular compound' of two or more similar, but distinct, peptides crystallizing together in definite proportions. In the Experimental Section of this paper we present evidence that substances made up of some of the same amino-acids as are in gramicidin, but present in different proportions, are to be found with it in tyrothricin.

As pointed out by us [Gordon *et al.* 1941], stoichiometric data do not justify conclusions about the relative positions of the various residues in the molecule, and we hope, should material be available, to throw light on the arrangement of the residues by a study of partial hydrolysis products [cf. Gordon *et al.* 1943*b*].

Gramicidin is the first naturally occurring peptide-like substance of this degree of complexity which is probably homogeneous, and for which the amino-acid composition has been ascertained with reasonable accuracy and completeness. Of the protamines, which appear to have similar molecular weights [cf. Svedberg & Pedersen, 1940], clupein, which has been most closely investigated, has been shown to be a mixture of substances fractionable by solvent precipitation of their methyl esters [Felix & Dirr, 1929; Felix & Mager, 1937]. Although it is certain that two-thirds of the residues of clupein are arginine residues, technical difficulties have in the past prevented accurate analysis of the monoamino-acids present. The hormone secretin appears to be a peptide of similar molecular weight [cf. Ågren, 1939]. Niemann [1939] has shown that, according to Ågren's analyses, some of its amino-acids occur in the proportions postulated by himself and Bergmann.

Apart from tyrocidine, which is found with gramicidin, few naturally occurring polypeptides of this order of molecular size have been accurately characterized. Systematic search will probably reveal more of them, and they may prove, as Kossel [1928] hoped, a valuable bridge between the very complicated proteins and their simple hydrolysis products. The study of such compounds will be particularly fruitful when they display physiological activity. The study of the partial hydrolysis products [cf. Gordon *et al.* 1943*b*] is likely at the present stage to give less equivocal results as to structural sequences in the molecule than the study of proteins containing hundreds of amino-acid residues. Also some of the partial hydrolysis products may retain physiological activity, and thereby throw light on its nature.

Dubos & Hotchkiss [1942] have reviewed biological aspects of the anti-bacterial action of gramicidin. The present work confirms that gramicidin is very unlike the average protein in amino-acid composition—notably in the absence of basic and acidic constituents and in the high proportion of tryptophan, leucine and valine. Lipmann *et al.* [1941] have discussed the possibility that the *d*-amino-acid residues play a part in its antibacterial action. Possibly the as yet unknown hydroxyamino compound has a special role, or the valine and leucine residues may have special inhibitory effects such as were noticed by Gladstone [1939] with these amino-acids in the free form in the nutrition of *Bacillus anthracis*. Unfortunately, Gladstone does not report experiments employing the *d*-isomers in the absence of the *l*-isomers. Perhaps, however, larger and more specific structural features of the molecule are concerned. Gramicidin might act in any of these ways if chemotherapeutic action depends on competitive inhibition of metabolic processes. Further study of its chemical structure along the above lines would seem to be a promising approach to this problem.

EXPERIMENTAL

Preparation of material for analysis—question of homogeneity

All samples of gramicidin were made by us from a Wallerstein preparation of tyrothricin. Four separate specimens were studied, the preparation of which we describe here. Before analysis all samples were dried to constant weight over H_2SO_4 and soda-lime in a vacuum desiccator at room temperature. N determinations were made by the Kjeldahl procedure after pre-treatment with red P and HI as recommended by Hotchkiss & Dubos [1941]. In our hands this procedure gave theoretical N values with acetyl-

tryptophan. Hotchkiss & Dubos record the N content of gramicidin as 14·8% and give the following optical data: $(\alpha)_D^{25°}$ +2·5° (ethanol, c=1·5), $(\alpha)_D^{25°}$ +5° (95% ethanol, c=0·4).

Preparation A. 4 g. of tyrothricin were worked up by the method of Hotchkiss & Dubos [1941]; the crude crystalline material was recrystallized once from acetone. After washing with ether the product weighed 117 mg. The whole preparation was hydrolysed for qualitative investigation of the amino-acids.

Preparation B. 1 g. of tyrothricin was worked up in the same way, the crude product was recrystallized thrice from acetone and washed with ether each time. Yield 19·9 mg. M.P. 226–227° (uncorr.). (Found: N, 14·6%.)

Preparation C was made from the combined mother liquors from the crystallization of preparations A and B, using the ethanol-ether precipitation of Hotchkiss & Dubos for preliminary purification. The first crop of crystals had $(\alpha)_D^{20°}$ −8·8° (ethanol, c=0·7). On recrystallization from acetone the product had $(\alpha)_D^{20°}$ −4·1° (ethanol, c=1·2). A further recrystallization yielded the final product, 18·5 mg., having $(\alpha)_D^{18·5°}$ +1·5° (ethanol, c=0·6). (Found: N, 13·0%.) In this, and the following preparation, washing of the crystals with ether was omitted, and this may have been responsible for the presence of the fatty material, which appeared as a separated layer after hydrolysis with acid. This fatty contaminant is presumably responsible for the lowered N content of preparations C and D.

Preparation D. This was made from the mother liquors from the recrystallization of preparation C. It did not readily yield crystals even after treatment with ethanol-ether. A purification by partition chromatography was attempted. It was found that gramicidin could be detected visually very readily owing to its fluorescence (presumably due to the tryptophan residues) on exposure to ultraviolet light. Trial experiments with silica gel chromatograms (using no indicator) showed that gramicidin can be made to move as a band. In the solvent system methanol-cyclohexane (the more polar phase being stationary, and absorbed in the gel), gramicidin scarcely moved at all on development. In aqueous systems, using as developing solvents 30% *n*-propanol-cyclohexane, 30% *iso*propanol-cyclohexane and 15% ethanol-cyclohexane, gramicidin moved as a band having R=>1, 0·5 and 0·2 respectively (same terminology as in previous paper [Gordon *et al.* 1943*a*]). The main preparation was then carried out on about 65 mg. of crude material, using 30% *iso*propanol-cyclohexane as the developing solvent. This caused the separation of three fluorescent fractions from the main band. One of these moved at R>1 (presumably fatty components), while the other two moved more slowly than the main fraction. This main fraction was run out of the column and collected separately; after evaporation of the solvent *in vacuo*, the preparation was taken up in ethanol and filtered to free it from silica which had escaped with it from the column. The ethanol was removed *in vacuo*, and the product was then allowed to crystallize from acetone. Crystallization was much slower than in previous preparations, taking 24 hr. for completion. The product, weighing 35 mg., had $(\alpha)_D^{20°}$ +3° (ethanol, c=1·1) and on recrystallization (an equally slow process) 18·3 mg. of the final product was obtained having $(\alpha)_D^{20°}$ +2° (ethanol, c=0·6). (Found: N, 12·9%.) Like preparation C, this material yielded a fatty layer on acid hydrolysis.

It seems therefore that some of the fatty material is so closely associated with the gramicidin that it resists chromatographic separation. The chromatographic purification, though eliminating the laevorotatory material noticed in the course of preparation C, does not give a really pure product, since, in addition to its slow crystallization, preparation D differs significantly in amino-acid composition from preparations B and C, having a lower content of valine and tryptophan, and a higher content of glycine, alanine and leucine than they have. This trend in composition of material obtained by working up the mother liquors (already noticeable in preparation C—cf. Table 6) suggests that, in addition to gramicidin, tyrothricin contains other components made up of some of the same amino-acids as the latter. In the analysis of none of these preparations have any qualitative differences in the amino-acids present been noted.

Although the chromatographic procedure used with preparation D did not lead to any dramatic purification, it seems likely that chromatographic methods may be of value for the quantitative extraction of gramicidin from tyrothricin preparations. The results mentioned here justify further study. From the data on simpler compounds given by Gordon *et al.* [1943*a*], gramicidin appears to distribute itself between solvent phases in a manner not inconsistent with its amino-acid composition.

Our overall yields of material judged to be rich in gramicidin have never amounted to more than 5% of the 5 g. of tyrothricin which we had at our disposal. This is rather less than the yields mentioned by Hotchkiss & Dubos [1941].

Antibacterial activity of the preparations

Dr F. C. Happold of Leeds University has kindly carried out bacterial growth inhibition tests [cf. Dubos & Cattaneo, 1939] on our preparations B, C and D, and reports:

'The samples of gramicidin have been tested for bactericidal or bacteriostatic action in tubes of beef infusion peptone broth (5 ml.). A 6 hr. culture of *Staphylococcus aureus* was inoculated into the experimental tubes and growth observed at 4, 5 and 6 hr. after inoculation. At 6 hr. a loop of each culture was spread over nutrient agar to give an additional comparison of the degree of growth. The gramicidin used was 200 μg./ml. in 2% ethanol and formed a stable suspension. A 1/10 dilution of this was also made in sterile distilled water so that when 0·2, 0·1 and 0·05 ml. of each of these two suspensions were added to the tubes of broth they gave concentrations of 40, 20, 10, 4, 2 and 1 μg./5 ml. medium.

'Preparations C and D were tested with a culture which was less rapidly viable than the one used with B. The result was even more dramatic. The

Table 3. *Antibacterial activity of gramicidin*

Preparation B	Growth at 4 hr.	5 hr.	6 hr.
Peptone infusion broth	+ + +	+ + + +	+ + + +
+ 0·2 ml. 2% ethanol	+ +	+ + + +	+ + + +
+ 1 μg. gramicidin	+	+ + +	+ + +
2 ,,	Tr.	+ +	+ +
4 ,,	Nil	?	+
10 ,,	Nil	Nil	? Tr.
20 ,,	Nil	Nil	Nil
40 ,,	Nil	Nil	Nil

alcohol controls and the infusion broth tube alone showed signs of growth at 6 or at 24 hr. When a loop of each tube was spread on agar all the gramicidin tubes were found to be obviously sterile.'

Analytical procedure

Following Hotchkiss [1941] we hydrolysed with HCl in aqueous acetic acid excluding air. After evaporation from alcoholic solution in a vacuum desiccator over H_2SO_4 and soda-lime, all our preparations (in Hysil test-tubes) were dissolved in 0·8 ml. of glacial acetic acid. 3·0 ml. of 6*N* HCl (which caused some precipitation) was added, the tube was evacuated, sealed, and heated for 24 hr. in a bath of refluxing 6*N* HCl. The precipitated material dissolved during the first $\frac{1}{2}$ hr. of heating.

The hydrolysate was prepared for chromatography as described by Gordon *et al.* [1943*a*]. A preliminary investigation, using the analytical technique described there, revealed that a shortened procedure could be used for separating the acetyl derivatives. This is illustrated in Table 4.

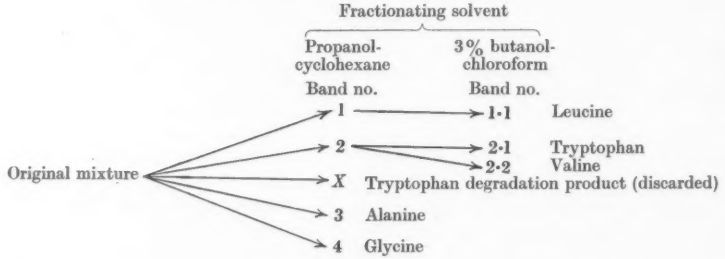
Characterization of gramicidin constituents

Preparation A (117 mg.) was fractionated according to the procedure described above with columns of a suitably increased diameter. The resulting fractions were worked up and characterized as described below. All M.P.'s are uncorrected unless otherwise stated. N determinations were made by the Kjeldahl procedure on samples of material in solution, which samples had been titrated with *N*/100 $Ba(OH)_2$ to determine the acid equivalent weights, using the N-free indicator phenolphthalein. For the N determination on acetyltryptophan, the titration mixture was evaporated to dryness *in vacuo* and treated with red P and HI before incineration, as in the N determinations on gramicidin. C, H determinations were carried out by Dr G. Weiler, Oxford.

(1·1) *Acetyl*-d-*leucine.* Successive recrystallizations from *n*-propanol-cyclohexane and from water yielded 20 mg. of product having M.P. 183° (not depressed on admixture of authentic acetyl-*d*-leucine, but depressed on admixture of acetyl-*l*-leucine), $(\alpha)_D^{19°}$ +17·2° (ethanol, *c* = 0·7). Synge [1939] records for acetyl-*d*-leucine M.P. 186–188°, $(\alpha)_D^{22°}$ + 23·2° (ethanol, *c* = 3·7). (Found: C, 55·1; H, 8·61; N, 8·0%; acid equiv. wt. 174. Calc. for $C_8H_{15}O_3N$: C, 55·5; H, 8·67; N, 8·1%; acid equiv. wt. 173.)

(2·1) *Acetyl*-l-*tryptophan.* Successive recrystallizations from chloroform-petrol and from water yielded 16 mg. of product having M.P. 178° (not depressed on admixture of authentic acetyl-*l*-tryptophan), $(\alpha)_D^{20°}$ +23·6° (ethanol, *c* = 0·5). Du Vigneaud & Sealock [1932] record M.P. 189–190° (corr.), and Gordon *et al.* [1943*a*] record $(\alpha)_D^{19°}$ +26·0° (ethanol, *c* = 1·1). (Found: C, 62·4; H, 5·68%. $C_{13}H_{14}O_3N_2$ requires: C, 63·3; H, 5·69%.) The material, recrystallized from $CHCl_3$, was further analysed as the molecular compound with $CHCl_3$ [cf. Gordon *et al.* 1943*a*]. (Found: N, 7·4%; acid equiv. wt. 358. Calc. for $C_{13}H_{14}O_3N_2.CHCl_3$: N, 7·7%; acid equiv. wt. 365·5.)

Table 4. *Scheme of fractionation*



In the propanol-cyclohexane column fraction 1 was run out with 5% propanol-cyclohexane. The remaining fractions were run out by development of the chromatogram with 30% propanol-cyclohexane. The qualitative appearance of the bands during the analysis of all four preparations was the same. Initial experiments indicated that fraction 1 required refractionation on a butanol-chloroform column to eliminate two components appearing as faint bands, running a little ahead of, and a little behind, the main acetylleucine band. Fractions 3 (alanine) and 4 (glycine) did not require further fractionation with butanol-chloroform.

(2·2) *Acetyl*-dl-*valine.* A determination of optical rotation on the whole fraction (44 mg.) gave $(\alpha)_D^{20·5°}$ 0 ± 0·3° (ethanol, *c* = 1·5). Gordon *et al.* [1943*a*] give for acetyl-*l*-valine $(\alpha)_D^{19°}$ +4·0° (ethanol, *c* = 2). On this basis the present material contains >92·5% of the racemic compound.

Two recrystallizations from $CHCl_3$ yielded a product having M.P. 145° (not depressed on admixture of authentic acetyl-*dl*-valine for which Synge [1939] records M.P. 144–146°). Acetyl-*dl*-*nor*valine [cf. Gordon *et al.* 1943*a*] appears

to have a much lower M.P. (Found: C, 52·2; H, 7·97; N, 8·6%; acid equiv. wt. 163. Calc. for $C_7H_{13}O_3N$: C, 52·8; H, 8·17; N, 8·8%; acid equiv. wt. 159.)

(3) *Acetyl-l-alanine.* Crystallization from ethyl acetate-benzene yielded 12 mg. of a product having M.P. 125° (not depressed on admixture of acetyl-*l*-alanine derived from gelatin), $(\alpha)_D^{18°} - 37·2°$ (ethanol, $c = 0·4$). For the product from gelatin Gordon *et al.* [1943*b*] record M.P. 126–127°, $(\alpha)_D^{18°} - 36·9°$ (ethanol, $c = 0·4$). (Found: C, 46·4; H, 6·92; N, 10·6%; acid equiv. wt. 130. Calc. for $C_3H_9O_2N$: C, 45·8; H, 6·87; N, 10·7%; acid equiv. wt. 131.)

(4) *Acetylglycine.* Crystallization from ethyl acetate yielded 2 mg. of a product having M.P. 200° (not depressed on admixture of authentic acetylglycine, M.P. 205–206° [cf. Curtius, 1884]. The M.P. was depressed to 180° on admixture of acetyl-*l*-glutamic acid). There was barely sufficient material for an accurate determination of N and of acid equivalent weight, so no C, H determination was carried out. Together with the M.P. and chromatographic data, however, these analyses provide reasonable identification of the substance. (Found: N, 11·2%; acid equiv. wt. 123. Calc. for $C_4H_7O_3N$: N, 12·0%; acid equiv. wt. 117.)

Absence of serine from gramicidin

Serine was used in the known mixture employed as a control to the quantitative experiments described below. It always gave a distinct band during analysis, travelling at 0·55 time the rate of band 4 on the 30% propanol-cyclohexane column. This band was never observed during analyses of gramicidin. With the latter a much fainter band moving at 0·3–0·4 time the rate of band 4 was occasionally observed. Whether this was due to the hydroxy-amino compound of gramicidin further investigation will show.

Fraction X (Table 4) was regularly observed in the control experiments as well as in those with gramicidin; separate experiments showed it to be a product derived from tryptophan.

The two minor components of fraction 1, shown as occurring in gramicidin hydrolysates, were not observed during analysis of the known mixture.

Quantitative analysis of gramicidin hydrolysate

6–9 mg. of gramicidin were used for a single analysis, on chromatogram tubes of 1 cm. internal diameter. 3 g. of silica was used in each column.

After an estimate of the relative proportions of the various constituents had been formed, control analyses were carried out on a known mixture of acetamino-acids and serine, containing an amount of each constituent nearly equal to that present in the samples of gramicidin analysed, the same hydrolysis conditions and analytical procedure being used. Apart from the differences mentioned above, the chromatograms were indistinguishable from those obtained with gramicidin. The constituents of the mixture and the percentage recoveries are shown in Table 5. The acetyl derivatives were used in preference to the free amino-acids since, in addition to being of ascertained purity, their hydrolysis simulated more closely the hydrolysis undergone by gramicidin.

Table 5. *Control experiments on gramicidin analyses*

Component of mixture	Recovery (%)
Acetyl-*d*-leucine	101, 101
Acetyl-*l*-tryptophan	66, 73
Acetyl-*dl*-valine	104·5
Acetyl-*dl*-alanine	98, 101
Acetylglycine	78, 63
dl-Serine	—

Control experiments showed the high valine figure to be due to contamination by acetyltryptophan which 'tailed' during the separation of fractions 2·1 and 2·2 with 3% butanol-chloroform. This was caused by using, throughout these experiments, a batch of gel which had been aged for too long under acid [cf. Gordon *et al.* 1943*a*]. No such 'tailing' was noticed on the propanol-cyclohexane columns made with the same gel. Accordingly, in the analyses shown in Table 6, the valine figures have been calculated from the titrations for fraction 2·2 *less* 6·8% of the titration for fraction 2·1. No correction has been applied to the leucine and alanine figures.

The low tryptophan recoveries are due to destruction of tryptophan in the course of hydrolysis, etc.; the low glycine recoveries presumably to incomplete extraction of acetylglycine from the acidified acetylation mixture by the 250 ml. of 17% butanol-chloroform used. The figures for glycine and tryptophan in Table 6 have accordingly been corrected assuming these recoveries, and must be regarded as semi-quantitative only. Reserve is particularly necessary in assessing the tryptophan figures, since the destruction during hydrolysis of tryptophan residues in combination in gramicidin may be different from that occurring when they are present in acetyltryptophan.

Table 6 shows the analytical results for preparations B, C and D corrected in this way.

Table 6. *Amino-acid composition of gramicidin preparations*

(N as % of total N)

Preparation ...	B	C	D
Leucine	20·0	20·4	21·6
Tryptophan	40·1–44·8	40·1–44·8	33·5–37·2
Valine	17·0*	16·6	15·6
Alanine	9·8	10·4	11·5
Glycine	5·0–6·2	5·7–7·0	6·6–8·0

* Maximum figure, owing to accidental contamination of the material for analysis by acetylvaline. This figure has not been incorporated in the mean values given in Table 2.

In compiling Table 2, the mean values of the determinations on preparations B and C have been used.

SUMMARY

Gramicidin has been subjected to acid hydrolysis, and the presence in the hydrolysate of *d*-leucine, *l*-tryptophan, *dl*-valine, *l*-alanine and glycine has been established.

A quantitative determination of these components has been made, and from this, in conjunction with the data of other authors, it seems probable that the gramicidin molecule contains the following 24 residues: 6 leucine, 6 tryptophan, 5 valine, 3 alanine, 2 glycine and 2 of an unknown hydroxyamino compound.

We are grateful to Dr R. J. Dubos and to Sir H. H. Dale, P.R.S., for making a supply of tyrothricin available to us, and to Dr B. C. J. G. Knight, whose suggestion led us to undertake this work. We wish to thank the Director and Council of the Wool Industries Research Association and the International Wool Secretariat for permission to publish this work.

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A Study of the Partial Acid Hydrolysis of Cow-hide Gelatin

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In a recent paper [Gordon, Martin & Synge, 1941] we described preliminary experiments aiming at the characterization of products of partial hydrolysis of proteins and through this at the elucidation of structural sequences in the intact protein molecule.

In the present paper we report further work in this direction, using partition chromatography [cf. Martin & Synge, 1941*b*; Gordon *et al.* 1943 *a*, *b*]. This procedure has made possible the semi-quantitative isolation of a number of peptides in the form of their acetyl derivatives. Peptide isolations have usually depended on the chance insolubility of a particular peptide or its derivative, and have given unknown yields. The new, more refined methods make possible a study of all the peptides of a given class occurring in a partial hydrolysate of a protein, and thus provide a more extensive picture than was formerly possible. The chromatographic procedure has been used simultaneously for rapid determination, on the micro scale, of the amino-acid composition of the peptide fractions isolated, an essential step too frequently neglected by workers in this field.

Apart from its value for the study of protein structure, this work may have applications in the field of serology, since Landsteiner [1942] has shown that the products of the partial hydrolysis of an antigenic protein can specifically inhibit its precipitin reactions with a homologous antiserum.

The present work was carried out with gelatin, since the absence of tryptophan, tyrosine and cystine from this material considerably simplifies the isola-

tive part of the investigation. This afforded us simultaneously a relatively simple check on current speculations as to the sequence of amino-acids in gelatin. The bearing of our experimental data on these speculations is discussed at the end of this paper.

Commercial gelatins are derived from the skin or bones of a number of species of animal, and are often mixtures in the biological sense. Because the parent collagens show significant differences in X-ray diffraction properties [cf. Astbury, 1940], it seemed particularly desirable to work with gelatin derived from a single source. The material used in the present work was a sample of cow-hide gelatin kindly placed at our disposal by Messrs Ross (Gelatines), Ltd., of Newlay, Leeds.

EXPERIMENTAL

(1) *Analytical methods*

Determinations of N (Kjeldahl), amino-N, free amino-acids (by ninhydrin) and tyrosine were carried out as previously [cf. Gordon *et al.* 1941]. The procedure used for determining monoamino-acids was as follows:

Determination of monoamino-acids. The chromatographic method previously described [Martin & Synge, 1941*b*] had, for the present work, been extended to deal with alanine, and in certain circumstances with glycine. It had further proved possible to differentiate valine and methionine from proline by determination of amino-N. The analysis was carried out as before [Gordon *et al.* 1943*a*] except that the series of refractionations with propanol-cyclohexane was omitted, the material from the butanol-chloroform column being titrated directly. 1% butanol-chloroform, followed by 17% butanol-chloroform after band 3 had run out, was used for development of the chromatograms. Phenolphthalein was used as indicator for the titrations.

The acetylalanine band was followed by that of acetylglycine. This was run out and titrated in the same way. As explained elsewhere [Gordon *et al.* 1943*a*], this titration represents glycine only if cystine, lysine, glutamic and aspartic acids are absent, otherwise, under these conditions, the acetyl derivatives of those acids move at about the same rate as acetylglycine. When the glycine band has moved some way down the column, any hydroxyproline present appears as a more slowly moving band. No attempt was made to run this out, but semi-quantitative estimates could be made on the basis of the colour and breadth of the band.

After titration with $Ba(OH)_2$ the proline-valine-methionine fraction was hydrolysed by refluxing for 5 hr. in 6*N* HCl at a volume of 10 ml. After this de-acetylation, excess HCl was removed *in vacuo*, and it was neutralized with Na acetate. Amino-N was then determined on the fraction. This made it possible to differentiate the proline from the valine and methionine. In the control experiment described below, where proline was separated from a mixture of amino-acids not containing valine or methionine, 0·232 mg. of proline-N in the fraction gave only 0·007 mg. amino-N. Similar experiments showed that under the same conditions valine and methionine gave 85–95% recovery of amino-N. This imperfect recovery, and the fact that in many experiments only very small amounts of valine and methionine were present, make it necessary to interpret the relevant figures with circumspection.

Influence of indicators. Methyl orange, phenolphthalein and baryta were always present in the final fractions prepared for amino-acid analysis (cf. § 6). As it was possible that these substances might give rise during hydrolysis to acid degradation products which might interfere with the chromatography, control experiments with regard to their decomposition were undertaken.

Methyl orange degradation products. 100 mg. of methyl orange were refluxed for 24 hr. in 6*N* HCl. Excess HCl was removed *in vacuo*. The resultant material was acetylated, and extracted with 17% butanol-chloroform as usual and one-fifth of this extract was taken for the chromatogram. This was run first with 1% and then with 17% butanol-chloroform as described above. During the first stage no acid band appeared. On development with 17% butanol-chloroform a faint band was seen to move down slightly behind the front of the new solvent. This was followed by a second very faint band which was estimated to move at a rate between that characteristic of glycine and that of hydroxyproline. Neither of these bands was strong enough to give a significant titration.

Phenolphthalein degradation products. 10 mg. of phenolphthalein were refluxed with acid, acetylated and extracted as above, and the whole of the extract put on the column. On development with 1% butanol-chloroform, a faint band moved down the column having $R=1{\cdot}0$. A second band was observed near the top of the column. On development with 17% butanol-chloroform this band moved down at $R=0{\cdot}15$. A third faint band moved at $R=0{\cdot}065$. Again none of these bands was of significant intensity.

Percentage recovery of amino-acids in a known mixture. Thus it appeared that indicators did not interfere with the analysis and a control experiment on amino-acid recovery was carried out. The amino-acids in the proportions shown in Table 1, together with 1 ml. of a saturated aqueous solution of methyl orange, 0·5 ml. of a 1% solution of phenolphthalein in ethanol and 0·33 ml. of *N*/3 $Ba(OH)_2$ were made up to 10 ml. in 6*N* HCl. The solution was refluxed for 36 hr. and after removal of excess HCl *in vacuo* the mixture was acetylated and extracted as described above.

One-tenth of the extract (corresponding to 1·10 mg. N in the original amino-acid mixture) was put on a column as above. The resultant analysis led to the recoveries shown in Table 1. The low

Table 1. *Percentage recovery in a known mixture of amino-acids*

Amino-acid	N as % of total N of mixture: Taken	Found	% recovery
dl-Phenylalanine	15·5	11·5	74
dl-Leucine	15·3	15·3	100
l-Proline	14·9	14·3	96
dl-Alanine	19·1	18·1	95
Glycine	16·6	13·6	82
l-Hydroxyproline	18·6	—	—
	100		

recovery of acetylglycine is explained by the incomplete extraction of this acid from the aqueous acetylation mixture. All figures given for glycine in the present paper have therefore been corrected assuming 82 % recovery.

The low recovery of phenylalanine was due to the use of gel containing traces of alkali. This question is dealt with by Gordon *et al.* [1943*a*]. The unsatisfactory gel used in the present experiment was also used in analysing the 'neutral peptide' fractions from the 4-day gelatin hydrolysate. Elsewhere in the present work gel giving satisfactory phenylalanine recoveries was used.

The above control experiment gives an indication of the accuracy of the analytical operations described in this paper.

(2) *Analysis of gelatin*

Monoamino-acids. Since the present investigation was concerned mainly with peptides of the monoamino-acids, an analysis for the total of each of these components was first carried out on a complete hydrolysate.

The gelatin was hydrolysed by refluxing for 24 hr. in 6*N* HCl. A colorimetric tyrosine determination on the hydrolysate showed some colour-producing substance to be present—this, however, was mainly associated with the small amount of insoluble humin in the hydrolysate—and no band corresponding to tyrosine was ever noticed on the chromatograms [cf. Gordon *et al.* 1943*a*].

The hydrolysate was analysed for constituent monoamino-acids. The results are shown in Table 2, together with our later figures for the same sample of gelatin [Gordon *et al.* 1943*a*] and those of the best previous determinations on gelatin. In converting these earlier figures to a N basis it was assumed that the N content of the gelatin (not recorded by Dakin or by Bergmann & Stein) was 18·3 % [cf. Beach & Teague, 1942].

Table 2. *Monoamino-acids of gelatin*

(N as % of total N)

Author ...	(1)	(2)	(3)	Others	
Phenylalanine	1·0	0·4	0·6	—	
Leucine isomers	3·9	3·9	4·1	—	
Valine	1·5	1·7	0	—	
Methionine		0·3	—	0·4	Beach & Teague [1942]
Proline	9·7	9·6	7·5	11·6	(4)
Alanine	9·0	8·3	7·5	—	
Glycine	—	—	26·0	27·0	(4)
Hydroxyproline	—	—	8·2	8·4	Bergmann [1935]

(1) Present determination.
(2) Gordon *et al.* [1943*a*].
(3) Dakin [1920].
(4) Bergmann & Stein [1939].

The difference between the phenylalanine value obtained here, and that obtained by our later procedure, shows clearly the necessity for the second fractionations with propanol-cyclohexane. In other cases our two analyses are in substantial agreement.

Methionine. Determinations on the present sample of gelatin, kindly carried out by Dr S. Blackburn of Torridon by the Baernstein volatile iodide procedure, gave a corresponding figure of 0·67 %. After the gelatin had been hydrolysed by 10*N* HCl at 37° for several weeks, this value had fallen to 0·14 %, suggesting demethylation. The possibility that methionine was oxidized to its sulphoxide by the H_2O_2 used in the manufacture of the gelatin should not be ignored.

Valine. The isolation, from the present sample of gelatin, of valine, in the form of its acetyl derivative, is described below (§ 6*d*). This is the first time valine has been shown by isolation to be a constituent of gelatin. Fischer, Levene & Aders [1902] obtained some evidence for its occurrence but left the question open pending more detailed investigation. Dakin [1920] asserted that it was absent. Dr R. Block (private communication) has obtained evidence for its occurrence in gelatin, using a modification of the procedure of Fromageot & Heitz [1939] [cf. Block & Bolling, 1941: we have not had access to this publication].

(3) *Course of hydrolysis*

The gelatin was hydrolysed in the same manner as before [Gordon *et al.* 1941], by allowing it to stand in 10*N* HCl at 37°, samples being removed at intervals for estimation of amino-N and free amino-acids. Fig. 1 shows a plot of these results. The large

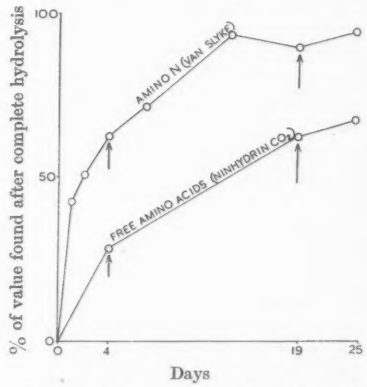


Fig. 1. Course of hydrolysis of gelatin in 10*N* HCl at 37°.

amount of glycine in gelatin makes it necessary to treat the amino-N figures with reserve. No correction for the glycine can be made because, although

the proper factor for the free amino-acid is well known, this is not the case when it is still in peptide linkage [cf. Van Slyke, 1911].

That the figure for free amino-acids had reached only a fraction of its final value when the figure for amino-N had almost reached 100% seems to indicate that the imino groups of proline and hydroxyproline are present, as previously suggested [Gordon *et al.* 1941], in peptide bonds resistant to acid hydrolysis at 37°.

The partial hydrolysates used for analysis were removed after 4 and 19 days. Further hydrolysis was stopped by removal of HCl by repeated evaporation with water *in vacuo*. The mixture was then diluted with water and stored at 0°. Fig. 1 shows that in the 4-day hydrolysate approximately one-third, and in the 19-day hydrolysate approximately two-thirds, of the residues had been converted to free amino-acids.

(4) *Preparation of 'basic' and 'neutral' fractions*

The electrodialysis of the partial hydrolysates was conducted in a diaphragm cell as previously described [Gordon *et al.* 1941], except that, in addition to the basic fraction, a neutral fraction was prepared. This consisted of the contents of the middle compartment of the cell after the first electrodialysis at *p*H 6. To ensure complete removal of basic and acidic material, the current was switched on again for a few minutes after the end compartments had been washed out, and refilled with distilled water. This was repeated a second time. Since some neutral material diffused out of the middle compartment during the preparation, the recovery of neutral amino-acids and peptides in this fraction was not quantitative. The amounts of the different substances lost depended on their different rates of diffusion, but only very slight changes in the relative amounts of the various molecular species left in the middle compartment, are to be expected.

In the case of the 'basic' fraction [cf. Gordon *et al.* 1941], the recovery should be quantitative.

The distribution of total N, amino-N, and free amino-acids in the partial hydrolysates and the fractions derived from these by electrodialysis is shown in Table 3. Amino-N and free amino-acids were determined before and after completely hydrolysing the material by refluxing for 24 hr. in 6*N* HCl. Comparison of these figures gives a measure of the amount of peptide material present in the different fractions.

The further analyses of the 'basic' and 'neutral' fractions are described separately below.

Table 3. *Analysis of fractions obtained by electrodialysis from partial hydrolysates of gelatin*

(N as % of total N of unfractionated gelatin)

	4 days' hydrolysis	19 days' hydrolysis
Unfractionated hydrolysate:		
Amino-N before complete hydrolysis	40·7	59·9
Amino-N after complete hydrolysis	67·0	67·0 (assumed)
Free amino-acids before complete hydrolysis	24·7	60·5
Free amino-acids after complete hydrolysis	87·0	87·0 (assumed)
Basic fraction:		
Total N	22·0	22·5
Amino-N before complete hydrolysis	6·3	7·6
Amino-N after complete hydrolysis	10·5	9·6
Free amino-acids before complete hydrolysis	2·3	5·5
Free amino-acids after complete hydrolysis	9·6	8·4
Neutral fraction:		
Total N	51·6	62·6
Amino-N before complete hydrolysis	25·3	37·8
Amino-N after complete hydrolysis	38·2	45·1
Free amino-acids before complete hydrolysis	16·2	45·7
Free amino-acids after complete hydrolysis	50·0	60·7

The above figures for free amino-acids are residues (1 mol. CO_2 evolved in presence of ninhydrin) expressed as N (1 atom) as % of the total N of the original unfractionated gelatin.

(5) *Investigation of the 'basic' fractions*

The 'basic' fractions obtained from the 4- and 19-day hydrolysates were analysed for monoamino-acids after complete hydrolysis, by the chromatographic method described above (§ 1). This experiment was similar to the analysis of the 'basic' fractions derived from various proteins [cf. Gordon *et al.* 1941], but owing to improved technique it could be carried out quantitatively. The amounts of the various amino-acids found are shown in Table 4. Assuming that these monoamino-acids are all linked as dipeptides with basic amino-acids, the yields from the 19-day hydrolysate account nearly quantitatively for the residues present in peptide linkage as determined by the ninhydrin procedure. Note also that the total amount of phenylalanine, leucine, valine and methionine residues bound in basic peptides after 4 days' hydrolysis is substan-

tially the same as after 19 days' hydrolysis. This is not, however, true for proline and alanine residues, and suggests that the bonds involving the former amino-acids are much more resistant to such hydrolysis than are those involving others such as proline and alanine.

Table 4. *Analysis of 'basic' fractions*

(N as % of total N of unfractionated gelatin)

Amino-acid	4 days' hydrolysis		19 days' hydrolysis	
Phenylalanine	0·05		0·04	
Leucine	0·29	0·56	0·29	0·59
Valine + methionine	0·22		0·26	
Proline	0·96	1·73	0·35	0·65
Alanine	0·77		0·30	
Total	2·29		1·24	
Residues in peptide linkage (moles as N by difference of free amino-acid determinations: cf. Table 3)	7·29		2·9	

(6) *Investigation of the 'neutral' fractions*

(*a*) *Chromatographic fractionation.* Preliminary experiments with synthetic acetyldipeptides [Gordon *et al.* 1943*a*] showed that these compounds are as readily separable by partition chromatography as are acetamino-acids, and that in general, for a given solvent pair, the acetylpeptides travel at rates intermediate between those for the acetyl derivatives of their constituent amino-acids.

In the present work on neutral peptides from gelatin, we have used successive fractionation on ethyl acetate-water and chloroform-butanol-water columns. This gives specially sharp separations of proline, whose acetyl derivative moves at the same rate as acetylvaline in chloroform-butanol, but moves more slowly than acetylalanine in ethyl-acetate. The proline peptides in partial hydrolysates of gelatin behave similarly, and are therefore specially amenable to study by the technique described here.

The 'neutral' fraction resulting from electrodialysis of the partial hydrolysate (corresponding to about 0·8 g. of gelatin) was evaporated to dryness *in vacuo* below 40°, acetylated, acidified and extracted with 17% butanol-chloroform as in the case of the monoamino-acid determination. The 4-day hydrolysate had 73 mg. N in the solution taken for the acetylation. In the 19-day hydrolysate 78·5 mg. N was present, 73% of which was obtained in the extract by this procedure.

The extracted material was fractionated on an ethyl acetate-water column of diameter 4·5 cm. Where the band was relatively fast-moving, and had 'run out' at the bottom of the column, the fraction was collected by evaporation of the solvent *in vacuo*. To obtain the slower moving fractions the column was pushed out of its tube after the developing solvent had run through, and each 'band' cut out immediately with a palette knife. Each fraction was immediately

Table 5. *Fractionation of 'neutral' material from 4-day hydrolysate*

	Fractionations			
		$R_{butanol\text{-}chloroform}$		
	$R_{ethylacetate}$	1% butanol	17% butanol	Final fractions
Acetylated extract	>1	0·5	—	I
	0·9	0·3	—	II
		0·15	—	III
		0·1	—	Discarded
		0·07	—	Discarded
	0·52	0·5	—	Discarded
		0·15	—	IV
		0·05	—	V
		—	0·24	Discarded
	0·3*	—	1·2	VI
		—	0·4	VII
	0·15	—	0·7	VIII
		—	0·45	IX
		—	0·23	X
	<0·08*	—	0·6	XI
		—	0·4	XII
		—	0·13	XIII

* These fractions left a residue insoluble in hot 17% butanol-chloroform which was not further fractionated.

Table 6. *Fractionation of 'neutral' material from 19-day hydrolysate*

	Fractionations			
		$R_{butanol\text{-}chloroform}$		
	$R_{ethylacetate}$	1% butanol	17% butanol	Final fractions
Acetylated extract	1·5	0·5	—	1
	1·35	0·5	—	2
		0·25	—	3
	0·9	0·15	—	Discarded
		0·10	—	4
		0·02	—	5
	0·6	0·15, 0·08	—	6
		—	0·4	Discarded
	0·26	—	0·7	7
		—	0·4	8†
	0·16	—	0·9	9
		—	0·3	10†
		—	0·1	11
	0·13	—	0·8	12
		—	0·15	13†
	0·05	—	0·8	Discarded
		—	0·5	14
		—	0·32	15
		—	0·19	16
		—	0·51	17
		—	0·42	18
		—	0·30	Discarded
		—	0·18	19
		—	0·13	20
		—	0·07	21
	0·04	—	0·4	22†
		—	0·07	23
	Slowest	—	0·46	24
		—	0·34	25
		—	0·17	26
		—	<0·12	Not analysed

eluted from the gel by thorough washing with alcohol on a Buchner funnel and the eluate evaporated to dryness *in vacuo*.

Every fraction so obtained was further fractionated on a chloroform-butanol-water column of suitable size and developed successively with solvent containing 1 and 17% of butanol v/v. The final fractions were collected in exactly the same way. For details see Tables 5 and 6.

In Tables 5 and 6 the final fractions, to each of which a number has been assigned, are tabulated. Several fractions were discarded, as too small. The symbol ⁀ between two fractions indicates grossly incomplete resolution of the corresponding bands; ⁀ indicates less serious mutual contamination, judged visually from the separation of the bands; while † indicates that the corresponding band was obviously due to more than one substance, being either very diffuse, or having the point of maximum concentration not near the leading edge of the band. Further fractionation on different chromatograms would have led to purer products, but was omitted because the present work was intended mainly as a preliminary survey.

The fractionation of the material from the 19-day hydrolysate was carried out in much greater detail than the fractionation of that from the 4-day hydrolysate, because during the initial fractionation on an ethylacetate column, the former resolved into a greater number of separate bands, between which 'cuts' could be made. Presumably the 19-day hydrolysate is a less complicated mixture. As seen in Table 5, the 4-day hydrolysate gave no resolution of bands having $R_{\text{ethylacetate}} < 0{\cdot}08$, the column above this level being uniformly pink. In the 19-day hydrolysate, a number of satisfactory 'cuts' could be made, and thus fractions 14–26 in the 19-day hydrolysate correspond to fractions XI–XIII in the 4-day hydrolysate. This in part explains the impossibility of interpreting stoichiometrically the amino-acid figures for fractions XI–XIII.

(*b*) *Amino-acid analysis of the final fractions.* Each of the final fractions was dissolved in ethanol, and a suitable aliquot was taken for amino-acid analysis. After evaporation of ethanol *in vacuo*, the material was dissolved in a little water and titrated with $N/100$ $Ba(OH)_2$, using phenolphthalein as indicator. This titration gave a measure of the number of equivalents of acetylpeptide and/or acetamino-acid present, and served as a check on the subsequent amino-acid analysis, thus:

Acetylglycylproline 1 equiv. —(hydrolysis and acetylation)→ acetylglycine 1 equiv. + acetylproline 1 equiv.

Acetylglycylglycine 1 equiv. → acetylglycine 2 equivs.

HCl was then added to the titration mixture so that the final volume (10 ml.) contained $6N$ HCl. The mixture was then refluxed for 24 hr. to effect complete hydrolysis. Monoamino-acids were determined as described in § 1 (see Table 7).

In any given fraction, the total quantity of each amino-acid is expressed as the percentage of the total N in the 'neutral' fraction of the corresponding partial hydrolysate of gelatin. The acid equivalents in the fraction before hydrolysis are expressed in the same units. The symbol * attached to an analytical figure indicates that in the fraction concerned the amino-acid in question occurred as its acetyl derivative and not as a peptide, and hence could be assumed to be in the free form in at least this amount in the partial hydrolysate. This is deduced from the analytical figures for the fraction in question, and is confirmed by (*a*) chemical characterization (see § 6*d*) and (*b*) the fact that the rate of travel on the chromatogram agrees with that expected for the particular acetamino-acid [cf. Gordon *et al.* 1943*a*].

The analyses showed no bands other than those corresponding to the known acetamino-acids and 'indicator artefacts', suggesting that the treatment with acid resulted in complete hydrolysis of all peptide material. In a few cases the 'glycine' band appeared to be complex, suggesting that lysine, glutamic or aspartic acids might be present. Peptides of lysine with either glutamic or aspartic acids should occur in the 'neutral' fraction resulting from electrodialysis.

The symbols 'ftr' (faint trace), +, etc., in Table 7 are relative to the total amount of material in the fraction submitted to analysis, and not with respect to the original gelatin.

Apart from fractions marked with an asterisk, in which single acetamino-acids predominate, the analyses in Table 7 suggest that the final fractions are still, in the majority of cases, complicated mixtures of acetylpeptides. Speculation as to the peptides present must be based, first, on the correspondence between the rates of travel of the band corresponding to the fraction on the different chromatograms and those of known acetylpeptides and acetamino-acids, and secondly on stoichiometry. Serine and threonine, for which the fractions have not been analysed, may be present in some of them, and may make interpretation yet more difficult.

The following fractions seem the only ones of reasonably obvious composition:

4-day hydrolysate

Fraction V. Mainly dipeptide glycine-leucine. The corresponding fraction 6 from the 19-day hydrolysate probably contains the same substance though more heavily contaminated.

Table 7. *Amino-acid analysis of final fractions*

	N as % of N of 'neutral' fraction of corresponding gelatin hydrolysate								
Fraction no.	Acid equivs. before hydrolysis (as N)	Phenyl-alanine + leucine + valine + methionine + proline	Phenyl-alanine	Leucine	Valine + methionine	Proline	Alanine	Glycine	Hydroxy-proline
				4-day hydrolysate					
I	0·09	0·09	0·09*	0	0	0	0	0	0
II	0·40	0·40	0	0·40*	0	0	0	0	0
III	0·77	0·70	0	ftr	0·63*	0·07	0·04	0·04	0
IV	0·25	0·28	0·14	0·12	0·02	→	0·05	0·16	0
V	0·79	0·66	0·08	0·58	ftr	→	0·08	0·59	+
VI	3·54	3·76	0·86	0	0·78	2·12*	0·28	0·50	+ +
VII	4·90	0·87	0	0·29	0·58	→	3·85*	0·82	0
VIII	0·36	0·41	0	0·18	0·05	0·18	tr	0·13	0
IX	0·23	0·23	0	0·08	0·04	0·11	0·10	0·07	0
X	11·7	—	fftr	ftr	fftr	→	0·61	11·0*	0
XI	0·60	0·78	0	ftr	0·12	0·66	0·23	0·23	+ +
XII	4·10	4·20	0·70	0	1·05	2·45	1·45	3·38	+ +
XIII	5·37	2·02	0	0·29	0·25	1·48	1·94	2·34	+ + + *
				19-day hydrolysate					
1	0·29	0·24	0·24*	tr	ftr	→	ftr	tr	ftr
2	0·47	0·41	0·41*	fftr	tr	→	ftr	tr	ftr
3	1·99	1·7	ftr	1·7*	fftr	→	fftr	ftr	fftr
4†	0·46	0·37	fftr	tr	0·36*	0·01	tr	tr	tr
5	0·32	0·18	0·06	0·07	0·05	ftr	0·06	0·11	0
6	1·13	1·06	0·17	0·67	0·14	0·08	0·21	0·61	+ +
7	0·42	0·42	0·04	0·20	0·17	0·01	0·12	0·11	+ +
8	10·4	—	0	fftr	tr	→	9·0*	1·15	fftr
9	8·15	6·79	0	fftr	0·39	6·4*	0·52	tr	+ +
10	0·56	0·30	0	0	0·20	0·10	0·17	0·21	+ +
11	0·64	0	0	0	0	0	0·05	0·64*	+
12	1·29	1·14	0	0	0·06	1·08*	ftr	tr	tr
13	23·3	—	0	0	fftr	→	tr	23·6*	0
14	0·19	0·12	0·02	0·03	0·04	0·03	0·02	0·04	+ + +
15	0·21	0·03	0	ftr	0·03	→	0·05	Lost	+ +
16	0·60	—	ftr	fftr	ftr	→	0·04	0·20	+ + +
17	0·06	0·06	0	tr	0·01	0·05	0·04	0·05	+
18	0·28	0·28	fftr	ftr	0·03	0·25	0·26	0·03	+
19	0·23	—	0	ftr	tr	→	0·07	0·06	+ + +
20	0·90	0	0	0	0	0	0·11	0·70	+ + +
21	1·17	—	0	0	fftr	→	0·06	ftr	+ + + *
22	1·20	1·05	0	0	0·09	0·96	0·22	0·81	+
23	2·76	—	0	fftr	tr	→	ftr	tr	+ + + *
24	0·14	0·18	0	0	0	0·18	tr	0·02	+ + +
25	0·27	0·22	ftr	0	0·03	0·19	0	tr	+ +
26	0·53	0·51	0	0	0·08	0·43	0·03	0·14	+ + +

† Minimum figures, as loss of unknown amount occurred in working up this fraction.

19-day hydrolysate

Fraction 17. Mainly tripeptide proline-alanine-glycine.

Fraction 18. Mainly dipeptide proline-alanine.

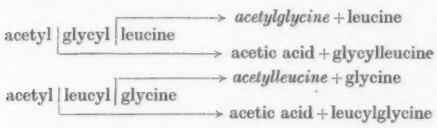
Fraction 22. A mixture of dipeptides proline-alanine and proline-glycine, with the latter predominating. The resolution of these substances seemed to have been achieved on the original ethyl-acetate-water chromatogram, with the result that fraction 18 was free from proline-glycine dipeptide, so some of fraction 22 was further fractionated on an ethylacetate-water column. Two bands were obtained. By cutting between these, two fractions were obtained—22*a* ($R_{\text{ethylacetate}} = 0{\cdot}063$) and 22*b* ($R_{\text{ethylacetate}} = 0{\cdot}050$). The band corresponding to 22*b* was much the stronger. Resolution of the bands did not appear to be perfect, and on amino-acid analysis of 22*b* it was found that 10% of alanine-proline dipeptide was still present with the glycine-proline dipeptide.

Fraction 24. Perhaps dipeptide proline-hydroxy-proline.

(*c*) *Determination of order of amino-acid residues in peptides.* Recrystallization and formal identification of the various acetyl peptides described above was impracticable owing to the small amounts at our disposal. The fractionations shown in Tables 5 and 6 were, for the sake of rapid working, each carried out on hydrolysates of less than 1 g. of gelatin.

We have, however, obtained some evidence as to the order of arrangement of the amino-acid residues in some of the peptides, a point on which the analyses given above yield no information. The following is a preliminary description of the chromatographic micro-method which we are developing for this purpose.

When an acetyldipeptide is subjected to partial hydrolysis, the initial splitting can occur in one of two ways, of which one leads to the formation of an acetamino-acid:


acetyl | glycyl | leucine
→ *acetylglycine* + leucine
→ acetic acid + glycylleucine
acetyl | leucyl | glycine
→ *acetylleucine* + glycine
→ acetic acid + leucylglycine

Thus acetylglycylleucine on partial hydrolysis should yield acetylglycine but no acetylleucine, and acetylleucylglycine *vice versa*. These acetamino-acids may be separated from the hydrolysis mixture by extraction and chromatography, and quantitatively determined.

Experiments were accordingly undertaken on synthetic specimens of these two acetyldipeptides [Gordon *et al.* 1943*a*], and in the light of the results of these, some of the fractions derived from gelatin were investigated.

(1) Acetyl-*dl*-leucylglycine was hydrolysed for 36 hr. in 10*N* HCl at 37°. An aliquot of this partial hydrolysate, corresponding to 7 mg. of acetyldipeptide was evaporated to dryness *in vacuo* below 40°, and stored overnight in a vacuum desiccator over H_2SO_4 and soda-lime to effect complete removal of acetic acid and excess HCl. The product was then analysed on a chromatogram, giving:

Acetylleucine	0·14 mol. per mol. of starting material
Acetylleucylglycine	0·13 mol. per mol. of starting material
And no acetylglycine	

(2) A similar experiment with acetylglycyl-*dl*-leucine gave:

No acetylleucine	
Acetylglycylleucine	0·40 mol. per mol. of starting material
Acetylglycine	0·26 mol. per mol. of starting material

(3) On the basis of these experiments a portion of fraction V (glycine-leucine dipeptide) was subjected to the same procedure, and gave:

Acetylleucine	0·10 mol. per mol. of starting material
Starting material	0·22 mol. per mol. of starting material
Acetylglycine	0·15 mol. per mol. of starting material

This suggests that the peptides glycylleucine and leucylglycine are simultaneously present in the partial hydrolysate.

(4). A similar experiment was carried out with fraction 22*b*. This gave:

No acetylproline	
Starting material	0·14 mol. per mol. of starting material
Acetylglycine	A faint trace

Exp. 4 suggests that we are dealing with acetylglycylproline, but is inconclusive owing to the small amount of acetylglycine formed. Exp. 2 shows that acetylglycine is comparatively resistant to hydrolysis under these conditions—and could therefore not have been formed in significant amount at any stage. The most plausible explanation is that the hydrolysis proceeds almost entirely at the bond between the acetic acid and glycine residues, thus rendering the method inapplicable. This result accords with the view of enhanced stability to acid hydrolysis of peptide bonds involving proline imino groups which we have postulated above (§ 3) to explain the course of hydrolysis of gelatin itself under the same conditions. We have not yet conducted experiments using synthetic acetylglycylproline to check this hypothesis.

The method described in outline above may prove of value for the location of free amino groups in more complicated peptides and in proteins. Extraction from acid aqueous solution by suitable organic solvents of the partial hydrolysis products of acetylated proteins or peptides should yield a mixture of acetylated amino-acids and peptides. Amino-acid analysis of a complete hydrolysate of this extract should give information as to the terminal amino-acids of the peptide chains and in the case of a substance consisting of a single unbranched acetylated peptide chain, e.g. Ac.$x_1.x_2.x_3$... should give the order of the residues directly, the quantities found being in the order $x_1 > x_2 > x_3$, etc.

The hydrolytic agent need not be an acid, but could be alkali, or, if rearrangement is out of the question, an enzyme. Other acyl groups than acetyl may also prove of value for the method.

(*d*) *Chemical identification of monoamino-acids as acetyl derivatives.* The amounts of acetamino-acids, as opposed to acetylpeptides, resulting from the fractionations described above, were adequate for complete chemical characterization. All were obtained as the *l*(+) optical stereoisomer.

All M.P.'s are given uncorrected. N determinations were by the Kjeldahl procedure. C, H determinations were done for us by Dr G. Weiler, Oxford.

Acetyl-l-phenylalanine. Crystallization from $CHCl_3$ of a fraction obtained from 10 g. of gelatin in the same way as fraction I yielded 22 mg. having M.P. 167–168° (not depressed on admixture of authentic specimen), $(\alpha)_D^{18°} + 45{\cdot}6°$ (ethanol, $c = 0{\cdot}5$). Jackson & Cahill [1938] record M.P. 170–171°, $(\alpha)_D^{25°} + 47{\cdot}6°$ (ethanol, $c = 1$). (Found: C, 63·4; H, 6·08; N, 6·84%; acid equiv. wt. 208. Calc. for $C_{11}H_{13}O_2N$: C, 63·7; H, 6·28; N, 6·77%; acid equiv. wt. 207.)

This isolation of phenylalanine in the optically active state by an acetylation and extraction procedure provides a check on the isolation of the racemic material from a wool hydrolysate [Martin & Synge, 1941*a*] confirming that racemization did not

take place in the course of isolation. Phenylalanine in wool must therefore exist in the racemic state, or be very readily racemized during hydrolysis by boiling acid.

Acetyl-l-leucine. Crystallization from $CHCl_3$ of a fraction obtained from 10 g. of gelatin in the same way as II yielded 40 mg. having M.P. 180° (not depressed on admixture of authentic specimen), $(\alpha)_D^{18.5°} - 20.0°$ (ethanol, $c = 1.3$). Martin & Synge [1941*a*] record M.P. 183–184°, $(\alpha)_D^{24°} - 22.6°$ (ethanol, $c = 3.3$). (Found: C, 56·2; H, 8·37; N, 8·0%; acid equiv. wt. 180. Calc. for $C_8H_{15}O_3N$: C, 55·5; H, 8·67; N, 8·1%; acid equiv. wt. 173.)

If *iso*leucine were also present in gelatin, acetyl-*iso*leucine should be found in the mother liquors from the above crystallization, which we have not further investigated. *Iso*leucine was stated by Dakin [1920] to be absent from gelatin.

Acetyl-l-valine. Crystallization from ethylacetate-benzene of a fraction obtained from 10 g. of gelatin in the same manner as III yielded 13 mg. having M.P. 155–156·5° (not depressed on admixture of authentic acetyl-*l*-valine), $(\alpha)_D^{20°} + 2°$ (ethanol, $c = 0.4$). Gordon *et al.* [1943*a*] record M.P. 164°, $(\alpha)_D^{19°} + 4.0°$ (ethanol, $c = 2$). The present material was evidently racemized to a considerable extent. (Found: C, 52·7; H, 8·00%; acid equiv. wt. 165. Calc. for $C_7H_{13}O_3N$: C, 52·8; H, 8·17%; acid equiv. wt. 159.) Our original N determination was lost and material did not suffice for accurate redetermination.

Acetyl-l-proline (*monohydrate*). Crystallization from wet ethylacetate of material from fractions 9 and 12 yielded 50 mg. having M.P. 80° (not depressed on admixture of authentic specimen), $(\alpha)_D^{19.5°} - 113.4°$ (water, $c = 1.3$, – rotation calc. for anhydrous compound). Martin & Synge [1941*a*] record M.P. 78°, $(\alpha)_D^{22°} - 114.0°$ (water, $c = 4.2$), and give references to previous determinations. (Found: C, 53·7; H, 7·07% (on anhydrous material.) Calc. for $C_7H_{11}O_3N$: C, 53·5; H, 7·01%.) (Found: N, 8·1; water of crystallization, 10·8%; acid equiv. wt. 170 (on hydrated material). Calc. for $C_7H_{11}O_3N.H_2O$: N, 8·0; water of crystallization, 10·3%; acid equiv. wt. 175.)

Acetyl-l-alanine. Crystallization from ethylacetate-benzene and ethylacetate successively of material from fraction 8 yielded 40 mg. having M.P. 126–127°, $(\alpha)_D^{19°} - 65.0°$ (water, $c = 0.7$), $[\alpha]_D^{18°} - 36.9°$ (ethanol, $c = 0.4$). Karrer, Escher & Widmer [1926] record M.P. 116°, $(\alpha)_D^{16°} - 45.6°$ (water, $c = 5$). Their material must have been grossly racemized as would be expected from their mode of acetylation. The acetyl-*l*-leucine described in the same paper was prepared in the same way, and was also largely racemic. (Found: C, 45·8; H, 7·00; N, 10·4%; acid equiv. wt. 136. Calc. for $C_5H_9O_3N$: C, 45·8; H, 6·87; N, 10·7%; acid equiv. wt. 131.)

Acetylglycine. Crystallization from ethanol of material from fractions 11 and 13 yielded 78 mg. having M.P. 202–203° (not depressed on admixture of authentic specimen). Curtius [1884] records M.P. 206°. (Found: C, 41·3; H, 6·01; N, 12·1%; acid equiv. wt. 120. Calc. for $C_4H_7O_3N$: C, 41·0; H, 5·98; N, 12·0%; acid equiv. wt. 117.)

(*e*) *Liberation of free amino-acids during the partial hydrolysis of gelatin.* From the figures marked with asterisks in Table 7 it is possible to deduce minimum figures for the amounts of free neutral amino-acids present in the hydrolysate after 4 and 19 days. Although losses of unknown extent occurred in the preparation by electrodialysis, of the 'neutral' fractions, they were probably similar for both the 4- and 19-day hydrolysates, and the *relative* amounts of the different amino-acids present were probably little affected in the course of isolation.

The figures are shown in Table 8, expressed as percentages of the number of residues of the same species present in the original gelatin. We base these latter figures on our present determinations shown in Table 2, and employ in addition the mean figure for glycine given in that table.

Table 8. *Free amino-acids in partial hydrolysates of gelatin*

(As % of total residues of same species present)

Amino-acid	4-day hydrolysate	19-day hydrolysate
Basic amino-acids*	32	78
Phenylalanine	5	40
Leucine	5	27
Valine + methionine	21	†
Proline	11	48
Alanine	23	63
Glycine	21	57

* The free basic amino-acids have been calculated from the data in Table 3, and are expressed as a percentage of the total arginine + histidine + lysine residues determined by Dakin [1920], assuming for gelatin an N content of 18·3%. No loss is associated with these figures for basic amino-acids, since the 'basic' fraction is obtained quantitatively by electrodialysis (cf. § 4).

† Not calculated owing to loss involved in isolation of fraction 4 (cf. Table 7).

It will be seen that the different amino-acids are liberated at significantly different rates as the hydrolysis proceeds.

DISCUSSION

Earlier work on the isolation of the products of partial hydrolysis of gelatin and other proteins will shortly be reviewed by one of us (R. L. M. S.). Here it will suffice to mention the isolation by previous workers of diketopiperazines corresponding to some of the dipeptides isolated in the present study. These former resulted either from enzymic hydrolysis or from treatment with hot dilute acids. Our hydrolysis with cold concentrated acid did not give rise to such products, which, had they been present, would have been isolated, since they are extractable by organic solvents [cf. Gawrilow & Lawrowsky, 1927], and would have raised the amino-acid content of the final fractions far above the acid equivalent determined by titration. We deduce that

these diketopiperazines are artefacts arising by condensation from the corresponding dipeptides.

Glycine-proline anhydride was isolated from enzymic digests by Levene [1910] and from acid hydrolysates by Gawrilow & Lawrowsky [1927] and Konuma [1938]. Gawrilow & Lawrowsky also obtained evidence for the occurrence of glycine-leucine anhydride. Dakin's [1920] proline-hydroxyproline anhydride more probably arose from condensation of free amino-acids in butanol than from a corresponding dipeptide, although the present work gives indications of a dipeptide of proline and hydroxyproline (fraction 24).

The results we have obtained are not inconsistent with the structure suggested by Astbury [1940] (cf. Bergmann [1935]) for gelatin. One-eighteenth of the total residues have not been allotted a definite place in this structure, and such peptides as we have found (leucylglycine *and* glycylleucine and peptides of bases with neutral amino-acids) which could not be fitted into the main arrangement do not total one-eighteenth of the whole. To reconcile our data with Astbury's structure for gelatin it is necessary to make the assumption that the acid hydrolysis of bonds involving these 'anomalous' residues proceeds more slowly than for the others, and we believe that the structure will be found to be much more complicated than Astbury suggests.

Apart from the arrangement of the small number of peptides for which we have obtained definite evidence, we have noted what may be an underlying structural principle concerning the arrangement of the amino-acid residues in the peptide chain; i.e. monoamino-acids with longer fatty side-chains (phenylalanine, leucine, proline, valine and methionine) apparently are not linked to one another in gelatin. This seems to be established: (1) by the almost complete absence of acetylpeptides giving bands running faster than acetylvaline on the chromatogram. From the behaviour of synthetic acetylleucylleucine [cf. Gordon *et al.* 1943*a*] we expect that bands due to this type of peptide will move faster than that of acetylvaline; (2) by the fact that in no fraction does the sum of the phenylalanine + leucine + proline + valine + methionine equivalents (cf. Table 7) exceed the number of acid equivalents before hydrolysis, as would be the case if two residues from this group of acids were in the same molecule.

Being a negative principle, it is less easy to establish, and we are tentative in drawing conclusions. But it seems that this spacing out of fatty residues along the peptide chain may determine some of the peculiar physical properties of gelatin, and further that the principle of distribution of amino-acids by physical classes [cf. Astbury, 1942] may be of general significance with regard to protein structure.

SUMMARY

1. An investigation has been made of the partial hydrolysis of cow-hide gelatin by 10*N* HCl at 37°.

2. 'Basic' and 'neutral' fractions have been isolated from the resulting mixtures by electrodialysis at *p*H 6.

3. Analysis of the 'basic' fractions strongly suggests that residues of basic amino-acids occur in gelatin directly linked to residues of the higher monoamino-acids.

4. Analysis of the 'neutral' material by acetylation followed by fractionation on silica gel chromatograms has led to the isolation of a number of lower peptides in the form of their acetyl derivatives.

5. By similar means the gelatin has been analysed quantitatively for its constituent monoamino-acids, and these have been formally identified in the form of their acetyl derivatives. *l*-Valine has been shown to be present, and the rates of liberation of the various amino-acids during the partial hydrolysis have been studied.

6. A chromatographic micro-method has been developed for the determination of the order of amino-acid residues in acetylpeptides, etc.

7. The relation of the present study to previous work on gelatin, and to current theories of gelatin structure is discussed. It is suggested that in gelatin the higher monoamino-acids do not occur in juxtaposition along the peptide chain.

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Fatty Acid Dehydrogenase in Adipose Tissue

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It has been established in recent years that adipose tissue in the body is not an inert site of fat deposition but a tissue with specific metabolic functions. This is proved by its respiratory activity [Mirski, 1942] and by its ability to deposit glycogen under suitable conditions [Hoffmann & Wertheimer, 1927; Tuerkischer & Wertheimer, 1942]. Further proof is given by experiments showing the dependence of various adipose functions upon innervation [Hausberger, 1937].

It might be assumed, in view of these results, that adipose tissue plays an important part in the deposition and mobilization of fat. Investigation of the fat metabolism of this tissue may therefore well prove of interest for the understanding of the deposition and mobilization processes.

The present investigation arises from the observation that aqueous extracts of adipose tissue decolorize methylene blue, the speed of the decolorization being enhanced when fatty acids are added. It was proposed, therefore, to prepare an active fatty acid dehydrogenase from adipose tissue and to study the properties of the isolated enzyme.

Towards the conclusion of this work, abstracts of papers by Lang [1939], Lang & Mayer [1939] and Lang & Adickes [1939], appeared in which the demonstration of fatty acid dehydrogenase in various sources is reported. As the original papers are not at present available to us, no comparison of methods is possible. The authors report that the dehydrogenase action is dependent on the availability of muscle-adenylic acid as coenzyme and that the activity is greater to high than to low fatty acids.

METHODS

Preparation of enzyme solution. Adipose tissue from the leg region and the abdominal cavity of rats is cut into small pieces with scissors and crushed with twice its weight of sand. The pulp thus obtained is extracted with 2 volumes of distilled water for ½ hr. at room temperature and centrifuged. The supernatant is freed of fat by filtration. The liquid is then dialysed through cuprophan for 6–20 hr. against running tap water. Adipose tissue of rabbits did not yield active extracts.

Boiled tissue juice was prepared by boiling 2 g. of adipose tissue pulp with 5 ml. water in a water bath for 30 min. The juice is filtered while hot.

Crude cozymase preparation was prepared according to Euler [1931]. Solutions obtained after cleavage of the lead precipitate with H_2S were used for the experiment.

Dehydrogenase activity estimations were carried out according to Thunberg. Test mixtures consisted of 0·1 ml. *M*/2 phosphate buffer *p*H 8·0, 0·1 ml. methylene blue (1/5000), with the following additions: 0·5 ml. enzyme solution, 0·1 ml. substrate (*M*/20) and 0·1 ml. coenzyme. When one of these constituents was not added it was replaced by distilled water. Fatty acids in the experimental mixture were neutralized with NaOH and brought to *p*H 8·0. In some cases, dehydrocholic acid was used for dispersion, as it had no influence on the dehydrogenase activity.

EXPERIMENTAL

As is shown in Table 1, extracts dialysed for 20 hr. did not cause decolorization of methylene blue even after the addition of fatty acids. However, when boiled tissue juice was added decolorization took place. The speed of decolorization was markedly enhanced by addition of fatty acids. Crude cozymase instead of tissue juice may be used with even better results. Neither tissue juice nor cozymase preparation caused decolorization when added to fatty acids but without enzyme. When enzyme solution boiled for 5 min. was used decolorization failed to take place even in the presence of coenzyme and substrate. The enzyme nature of the dehydrogenase is thus established.

Table 1. *Enzymic reduction of methylene blue*

Addition to enzyme soln.	Time of decol. min.
None	No decol.
Stearate	,,
Boiled juice	70
Boiled juice + stearate	30
Crude cozymase	90
Crude cozymase + stearate	12
Boiled enzyme + crude cozymase + stearate	No decol.
Boiled juice + stearate	,,
Crude cozymase + stearate	,,

In view of the report of Lang & Mayer [1939], experiments were undertaken to examine whether muscle adenylic acid can replace boiled tissue juice or crude cozymase as coenzyme. This was found to be the case. Inosinic acid can also act as coenzyme. Yeast adenylic acid did not display coenzyme activity.

Table 2. *Influence of coenzyme on reduction of methylene blue*

Addition to enzyme soln.	Time of decol. min.
None	No decol.
Palmitate	,,
Muscle adenylic acid (20 mg. %)	120
Muscle adenylic acid (20 mg. %) + palmitate	3
Inosinic acid (20 mg. %)	36
Inosinic acid (20 mg. %) + palmitate	3
Yeast adenylic acid + palmitate	No decol.

As is shown in Table 2, although inosinic acid can act as coenzyme, the use of adenylic acid is preferable, as inosinic acid itself caused comparatively rapid decolorization even in absence of substrate. Diphosphopyridinenucleotide does not seem to be involved, since adipose tissue extracts capable of serving as coenzyme of fatty acid dehydrogenase could not activate lactic dehydrogenase of muscle. In addition to coenzyme, inorganic phosphate must be present for this dehydrogenase action. When the phosphate buffer is replaced by bicarbonate, dehydrogenation is comparatively slow, as is shown in Table 3.

Table 3. *Effect of phosphate on dehydrogenation*

Addition to enzyme soln.	Time of decol. min.
Adenylic acid + phosphate	90
Adenylic acid + phosphate + palmitic acid	4
Adenylic acid + 1·3% bicarbonate	110
Adenylic acid + 1·3% bicarbonate + palmitic acid	75

p*H optimum of fatty acid dehydrogenase*

In determining the optimum *p*H for the dehydrogenase, difficulty was encountered in keeping the fatty acid in suspension below *p*H 8. This was overcome by the use of Na dehydrocholate as dispersing agent. Stearic acid was dispersed in 0·5% Na dehydrocholate and the reaction brought to the required *p*H with NaOH. By this means a comparatively stable suspension could be obtained at as low a *p*H as 6·0. Controls containing the same amount of Na dehydrocholate were done. The results are shown in Fig. 1, which demonstrates a clear optimum of dehydrogenase action at about *p*H 8.

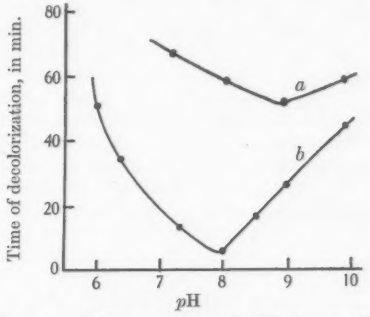

Fig. 1. *a*, without substrate. *b*, with stearic acid.

Substrate specificity

The activity of the enzyme towards fatty acids of different chain lengths, with even and uneven number of carbon atoms, as well as towards other substrates, was examined. A crude cozymase preparation was used in these experiments as coenzyme. The results are summarized in the following table.

Table 4. *Specificity of dehydrogenase*

Addition	Time of decol. min.
Enzyme + no substrate	90
+ stearate	2
+ palmitate	3
+ caprinate	10
+ capronate	10
+ valerate	25
+ butyrate	33
+ oleate	15
+ succinate	5
+ lactate	30
+ glycerophosphate	18
+ lecithin (sheep brain)	10
+ cephalin (sheep brain)	12
No enzyme + lecithin	>180
+ cephalin	>180
+ oleate	>180

The table shows clearly that with diminishing fatty acid chain length the enzyme activity decreases. The unsaturated fatty acid oleic acid is dehydrogenated much more slowly than the corresponding saturated acids. Also phospholipins are

dehydrogenated by the enzyme. Separate experiments confirmed that with phospholipins, as with fatty acids, the same coenzyme and inorganic phosphate are needed for dehydrogenase action. The optimal *p*H for fatty acid was found to be the same as for phospholipins. Of other substrates tested, succinate alone was found to be dehydrogenated at a rate comparable with that of the fatty acids.

In order to ascertain whether the fatty acid dehydrogenase was able to act on neutral fat, a dispersing agent other than fatty acid had to be employed. A suspension of neutral fat in 1 % lauryl alcohol sulphonate was used. As Table 5 shows this agent had no inhibiting effect on fatty acid dehydrogenation. Nevertheless, no decolorization of methylene blue was observed with a suspension of neutral fat.

Table 5. *Action of dehydrogenase on neutral fat*

Addition to enzyme soln.	Time of decol. min.
No substrate + coenzyme	75
Palmitate + coenzyme	4
Palmitate + coenzyme + lauryl alcohol sulphonate	4
Neutral fat in lauryl alcohol sulphonate + coenzyme	>120

Inhibitors of dehydrogenase action

In view of the fact that inorganic phosphate was necessary for dehydrogenase action, a number of poisons of phosphorylating reactions were examined as to their effect on fatty acid dehydrogenation. Other dehydrogenase poisons were also tested. In no case could an inhibiting action of these substances be observed. The substances and concentrations tested were: monoiodoacetate *M*/100, phloridzin *M*/100, NaF *M*/50, maleic acid *M*/200, pyrophosphate *M*/200 and benzoate *M*/100.

These results, as well as the impossibility of demonstrating any decrease in inorganic phosphate during dehydrogenase action, seem to exclude the possibility that the activating action of phosphate on the dehydrogenase involves phosphorylation.

The finding that maleate and pyrophosphate failed to inhibit decolorization is evidence against the identity of fatty acid dehydrogenase with succinic dehydrogenase.

Distribution of enzyme

An enzyme which desaturates fatty acids could be demonstrated in liver, muscle, heart, testes, brown fat as well as in adipose tissue. No activity could be demonstrated in brain. With these tissues, phospholipins were dehydrogenated at the same speed as fatty acids, in contrast to adipose tissue. Activity in these tissues was not as clearly demonstrated as in adipose tissue. This was due to the presence of other active dehydrogenases with their substrates, which caused decolorization in the absence of fatty acids. In order to remove all hydrogen donators prolonged dialysis (at least 24 hr.) was necessary. This treatment, however, also weakened the fatty acid dehydrogenase. Better results were obtained when the cut organs were washed with 0·9 % NaCl and then extracted with distilled water, followed by dialysis for 4–6 hr.

Influence of oxygen on dehydrogenase

Attempts to demonstrate dehydrogenase activity by measuring O_2 consumption in the presence of methylene blue were unsuccessful. This finding is explained by the fact that the enzyme mixed with coenzyme and substrate is inactivated in the presence of O_2. If, after mixing all components of reaction, the mixture is allowed to stand at room temperature in the air for 10 min. before evacuating, no activity can be observed even with Thunberg's method.

SUMMARY

1. A fatty acid dehydrogenase has been demonstrated in extracts of adipose tissue. Its activity depends on the presence of a coenzyme, adenylic acid, and inorganic phosphate.

2. The optimum *p*H of the dehydrogenase is 8·0.

3. Activity is highest with the natural, long chain, fatty acids and decreases with diminishing chain length.

4. The dehydrogenase also oxidizes phospholipins. Of other substrates tested only succinate was dehydrogenated at a rate comparable to palmitic and stearic acid. Neutral fat does not seem to be attacked.

5. Of tissues other than adipose tissue, liver, muscle, heart and testes, were proved to contain fatty acid and phospholipin dehydrogenase.

6. Monoiodoacetate, phloridzin, NaF, maleic acid, pyrophosphate and benzoate at fairly high concentrations do not affect the enzyme.

7. Exposure of the reaction mixture to O_2 caused rapid inhibition of the enzyme activity.

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Differences in Calcification between the Humerus and Femur of Young Rats receiving High Calcium, Low Calcium and Stock Diets

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The calcification of bones in both normal and pathological conditions, especially in rickets, is frequently described in terms of ash content in preference to histological structure. The methods used in the determination of bone ash vary little from one laboratory to another as regards the procedures for the extraction of the fat and the ashing of the bones, but the choice of the particular bone or bones to be examined is a matter of individual preference. Chick & Roscoe [1926] ashed femur, tibia and fibula together in the determination of the ratio mineral ash/organic residue as a rachitogenic index. Kon & Henry [1935], in carrying out prophylactic estimations of vitamin D, determined the percentage ash from one femur and one humerus together for each rat. Other workers have chosen one bone only as sufficiently representative, e.g. the tibia [Schultzer, 1931] or the femur [Bethke, Kick & Wilder, 1923]. In any of these procedures the final value for each rat is obtained from a single determination or from duplicate determinations made on right and left bones. Bethke *et al.* [1923] ashed separately the humeri and femora of each rat and obtained an average value from separate analyses of bones from different parts of the body.

In this laboratory it has been customary to follow Bethke's technique, and after many determinations it became apparent that these two bones from rats receiving a rickets-producing diet showed a difference in the degree of calcification, and that this difference increased with an increase in the severity of the rickets. Coward, Kassner & Waller [1938] have published a series of bone ash values from rats receiving a diet deficient in calcium. These values were all much lower than normal even with ample supplements of vitamin D. Their results show that in all groups the percentage ash was at a level usually associated with rickets but that there was little difference between humerus and femur. To study this point further a detailed analysis of all bone ash values which had been obtained in this laboratory was undertaken.

COLLECTION OF DATA

Data have been obtained over a period of several years, and include results already published. The percentage ash in the dry fat-free humerus was chosen arbitrarily as the basal value, and the results have been arranged in order of magnitude with respect to this datum, irrespective of sex of the rat, and of the dose of vitamin D given. The difference (d) between the percentage ash in the humerus and femur was related to the percentage ash in the humerus (H) by means of the expression $(d \times 100)/H$.

EXPERIMENTAL

All the animals were albino rats from the inbred colony of Wistar strain, maintained on a stock diet [Coward, 1938]. The young rats were weaned at 23 days, at an average weight of 40–45 g.; the test period was 5 or 6 weeks, except in one early experiment of only $4\frac{1}{2}$ weeks; four rats out of the total of 38 on stock diet were fully grown. At the end of the test period, both humeri and both femora were dissected out and the ash content determined on the dry fat-free bones, the right pair being taken from each rat. In two early experiments both humeri and both femora were ashed together, but this was not the usual procedure.

RESULTS

A. *The influence of diet*

Evidence was available from three different types of diet:

(*a*) Rachitogenic diet of the high Ca and low P type, Steenbock 2965 [Steenbock & Black, 1925]: 194 rats.

(*b*) Low Ca diet, the 'poorer class' diet described by Coward *et al.* [1938]: 109 rats.

(*c*) Stock diet as used in the rat colony: 38 rats.

The results, which for convenience have been grouped in ranges of four according to the percentage ash of the humerus, are shown in Table 1 and represented graphically in Fig. 1. The rats receiving stock diet, being so few in number, have been included in a single group in the table. In severe rickets the difference in calcification between humerus and femur is clearly shown. This difference decreases as the state of calcification improves. On the other hand, there is no difference between the two bones of rats receiving either stock diet or low Ca diet.

All the evidence available related to differences between humerus and femur from rats which had received the rachitogenic diet + vitamin D, so an experiment was carried out to determine the effect of the unsupplemented diet. It has been shown [Bourdillon, Bruce, Fischmann & Webster, 1931; Bacharach, 1940; Coward & Kassner, 1941] that the variation between litters is greater than the variation within the litter for many types of biological response. In the figures so far quoted no account has been taken of litter-mates nor indeed of any variable other than the percentage ash in the dry fat-free humerus. Therefore in this experiment comparisons were made of litter-mates subjected to the different conditions.

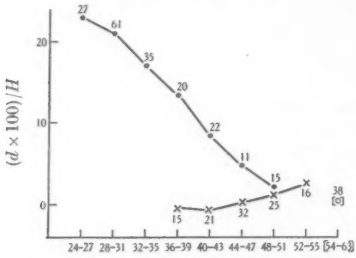

Fig. 1. The relation between the ash content of the humerus and the difference in calcification of humerus and femur of rats receiving three types of diet. •—• Rachitogenic diet. ×—× Low calcium diet. [o] Stock diet. Small figures give the number of animals.

39 rats were divided into three litter-mate groups of six bucks and seven does. Group 1 received stock diet; group 2 received the rachitogenic diet with 80 i.u. vitamin D_2 per week, this being a dose greatly in excess of the prophylactic requirement for young rats of our colony; group 3 received the rachitogenic diet only.

As comparatively few figures are available in the literature for individual responses in bone calcification, full protocols as well as the average values for this test are presented in Table 2. The regularity of response in these three groups, where maximum effects are being measured, is the outstanding feature of this table. The range of variation in the percentage ash of the humerus in all three groups is lower than that usually found among rats receiving the rachitogenic diet with a supplement of vitamin D insufficient to give complete calcification. Particularly is this the case for group 3, for under these conditions of diet rickets develops with regularity.

In the rachitic bones from group 3 in which the calcification is very poor (28·11 % ash in the humerus), the difference between humerus and femur is striking and is clearly significant. On a complete diet there is a difference in calcification between the humerus and femur, $t_d = 2{\cdot}10$, P = very slightly $> 0{\cdot}05$ for group 1. The calcification in group 2 is less than that in group 1, and the difference between the humerus and femur, though small, is significant, $t_d = 3{\cdot}55$, P = much $< 0{\cdot}01$. The inability of vitamin D alone to correct the faulty calcification brought about by a rachitogenic diet of the high Ca and low P type has often been recognized and is generally attributed to the limiting effect of the P [Hume, Pickersgill & Gaffikin, 1932].

It should also be noted that when the ash is calculated on the basis of mg./100 g. body weight the calcification in the group receiving the rachitogenic diet alone is very much less (54·4 mg. ash/100 g.) than that of the rats receiving either stock diet (130·8 mg. ash/100 g.) or the rachitogenic

Table 1. *The absolute and percentage differences in ash content of humerus and femur of rats receiving three types of diet and the standard deviations of those differences*

Range of % ash in the humerus	Mean difference 'd' between the % ash in the humerus and femur			Standard deviation of 'd'			Mean difference 'd' expressed as % $(d \times 100)/H$			Standard deviation of $(d \times 100)/H$		
	Rachitogenic diet	Low Ca diet	Stock diet	Rachitogenic diet	Low Ca diet	Stock diet	Rachitogenic diet	Low Ca diet	Stock diet	Rachitogenic diet	Low Ca diet	Stock diet
20–23	(3·04)*	—	—	—	—	—	(13·5)*	—	—	—	—	—
24–27	6·22	—	—	1·38	—	—	23·0	—	—	5·08	—	—
28–31	6·29	—	—	1·77	—	—	20·9	—	—	5·85	—	—
32–35	5·77	—	—	2·09	—	—	17·0	—	—	6·17	—	—
36–39	5·06	−0·09	—	2·39	1·69	—	13·4	−0·3	—	6·32	4·41	—
40–43	3·49	−0·24	—	2·40	2·06	—	8·4	−0·5	—	5·81	4·93	—
44–47	2·25	+0·15	—	2·88	1·26	—	4·9	0·3	—	6·35	2·70	—
48–51	1·09	+0·58	—	1·33	1·42	—	2·2	1·2	—	2·69	2·81	—
52–55	(0·63)†	+1·47	—	—	1·14	—	(1·2)†	2·7	—	—	2·06	—
54–63	—	—	0·11	—	—	0·79	—	—	0·2	—	—	1·36

* Two rats only. † One rat only.

The number of animals in each group varies from 11 to 61 and is given in Fig. 1.

Table 2. *Full protocols of individual responses as regards bone ash and body weight of rats receiving stock diet or rachitogenic diet with and without added vitamin D_2*

Group	Diet	Litter	Sex	% ash in humerus *H*	Difference in % ash between humerus and femur *d*	Difference expressed as % $(d \times 100)/H$	Body weight: At start g.	Body weight: At end g.	Increase in wt. g.	Total ash from both bones expressed as mg./100 g. body weight
1	Stock	9700	♀	56·40	−0·24	−0·4	42	111	69	141·4
		9702	♂	57·28	0·70	1·2	45	173	128	120·3
			♀	55·91	−0·72	−1·3	46	124	78	145·1
		9705	♀	57·14	−0·23	−0·4	46	138	92	139·6
		9715	♂	56·52	−0·06	−0·1	46	155	109	119·5
			♀	59·06	1·23	2·1	44	144	100	141·0
		9720	♂	54·50	0·74	1·4	48	153	105	121·4
			♀	57·85	1·18	2·0	42	131	89	129·1
		9722	♂	57·50	1·49	2·6	46	158	112	125·5
			♂	58·33	0·04	0·1	48	175	127	122·7
		9728	♀	58·65	0·78	1·3	46	127	81	134·3
		9744	♂	57·41	2·58	4·5	48	180	132	122·7
			♀	57·14	−0·45	−0·8	41	128	87	138·2
		Average		57·21	0·54	0·94	45·2	145·9	100·7	130·8
		S.D.		1·20	0·93	1·63	—	—	—	9·36
2	Rachitogenic plus excess vitamin D_2	9700	♀	51·32	1·80	3·5	46	77	31	118·7
		9702	♂	49·78	0·66	1·3	49	83	34	122·8
			♀	51·45	0·87	1·7	44	84	40	124·9
		9705	♂	50·63	0·40	0·8	50	98	48	119·1
		9715	♂	50·13	0·86	1·7	50	107	57	105·6
			♀	49·75	1·67	3·4	43	71	28	113·0
		9720	♂	49·83	4·55	9·1	45	70	25	109·6
			♀	49·34	2·65	5·4	47	66	19	127·9
		9722	♂	49·06	0·55	1·1	47	96	49	105·0
			♀	52·73	0·63	1·2	45	83	38	121·2
		9728	♀	51·92	1·19	2·3	44	59	15	144·7
		9744	♂	48·64	1·12	2·3	49	70	21	126·9
			♀	48·40	−0·69	−1·4	41	64	23	122·7
		Average		50·23	1·25	2·49	46·2	79·1	32·9	120·2
		S.D.		1·31	1·27	2·55	—	—	—	10·62
3	Rachitogenic	9700	♀	27·37	8·91	32·6	43	54	11	48·3
		9702	♂	28·50	6·91	24·2	50	68	18	52·2
			♀	27·57	4·42	16·0	43	56	13	56·4
		9705	♂	27·16	6·83	25·1	45	53	8	60·6
		9715	♂	28·15	6·86	24·4	45	53	8	57·0
			♀	28·75	5·58	19·4	44	52	8	57·9
		9720	♂	27·92	6·95	24·9	48	63	15	54·9
			♀	29·08	5·74	19·7	46	56	10	55·5
		9722	♂	29·25	7·31	25·0	48	63	15	51·6
			♀	27·64	5·17	18·7	46	61	15	53·1
		9728	♀	29·43	7·21	24·5	46	59	13	57·1
		9744	♂	28·21	8·92	31·6	49	65	16	51·1
			♀	26·39	6·99	26·5	41	51	10	51·8
		Average		28·11	6·75	24·05	45·7	58·0	12·3	54·4
		S.D.		0·89	1·30	4·77	—	—	—	3·41

diet + vitamin D (120·2 mg. ash/100 g.). The increase in body-weight is considerably less for rats on the rachitogenic diet than for rats on stock diet, even with excess of vitamin D. On the rachitogenic diet alone, this increase was very small from the start, and during the last 2 weeks a slight decrease took place.

B. *Distribution of calcification in the bone*

In the rachitic bone the metaphyses are greatly affected while the changes in the shaft are smaller and proceed at a slower rate during the development of the lesions [Hess, Berliner & Weinstock, 1931]. This suggested that the relatively small total ash content of the femur of a rachitic rat might be due entirely to the relatively large metaphyses. The weights and ash contents of the ends and shaft of the bones were therefore determined separately.

Left bones representative of each of the three diets (stock diet, low Ca diet, rachitogenic diet) were taken. The division of each bone into three, a shaft and two ends, was chosen arbitrarily but the cuts were always made as nearly as possible in the same places—in the femur just below the lesser trochanter and just above the condyles, in the humerus at the surgical neck and just below the supratrochlear fossa. For each bone the shaft was weighed alone and the two ends together. The results are shown in Table 3. Calcification is distributed in approximately similar proportions between shaft and ends in both humerus and femur for groups 2, 3 and 4; that is to say for rachitogenic diet + vitamin D_2, low Ca diet and stock diet, the humerus and femur show about the same degree of calcification, although the level varies with the diet, and so do the ends of both humerus and femur. In contrast to this, these bones from rats which have received the unsupplemented rachitogenic diet differ from one another both as regards the distribution of calcification between shaft and ends and also as regards absolute values for ash contents. The figures for rachitic rats show a difference between the two bones even when the shafts and ends are considered separately. The relatively low ash content of the whole femur was thus not entirely due to the fact that its metaphyses were relatively large, though it was partly due to this factor since the percentage difference was larger for the whole bone (6·68) than for either the shaft (4·60) or ends (4·27).

The fall in the ash content of the bones of the rachitic rats was most marked in the femur and particularly in the ends of the femur. This is perhaps due to the strain placed upon this bone because the weight of the rat is carried mostly on its hind legs.

In view of the many sources of possible error both in cutting dry fat-free bones and in ashing very small pieces of bone it should be emphasized that the figures in Table 3 represent only a general state of affairs. When the percentage ash in the whole left bone was calculated from that of the two parts and compared with the percentage ash previously determined for the corresponding right bone, the agreement in nearly all cases was good, showing that the figures in the table are representative of the bone both as regards relative proportion of parts and as regards their calcification.

These results suggest that, as is the practice in both the X-ray and the line test techniques, one part of one bone only might, with advantage, be used in the assay of vitamin D by the bone ash method. The difference in ash content between a severely rachitic bone and one showing the maximum calcification brought about by vitamin D alone, is 19·89 % for the humerus alone, 26·06 % for the femur alone, 22·98 % for the average of the humerus and femur and 28·31 % for the ends of the femur alone. These figures suggest that by using for the assay only the ends of the femur, which show the large response to vitamin D, an increase in the range of calcification is secured and hence a steeper curve of response and one which might be more sensitive for low doses. But further work is required, since the data here reported are insufficient to establish the point.

Table 3. *Ash content of ends as compared with whole bone and comparison between these portions in femur and humerus*

Group	No. of animals	Diet	Weight of ends / Total weight × 100		% ash in whole bone (calculated)		Difference in % ash in humerus and femur		
			Humerus	Femur	Humerus	Femur	Shaft	Ends	Whole bone
1	8	Rachitogenic	51	61	29·40	22·72	4·60	4·27	6·68
2	6	Rachitogenic + vitamin D	47	49	49·82	48·23	2·66	−0·27	1·59
3	10	Low Ca	46	45	46·70	45·61	2·87	−0·88	1·09
4	7	Stock	42	41	57·85	56·19	2·75	0·36	1·66

C. *Influence of sex*

In the young female rat ossification proceeds more rapidly than in the male [Cox & Imboden, 1936]. Donaldson [1924] gives figures showing that

Table 4. *The influence of sex on bone ash content*

Diet	Range of % ash in the humerus	No. of animals ♂	No. of animals ♀	Average value of d ♂	Average value of d ♀	Average value of $(d \times 100)/H$ ♂	Average value of $(d \times 100)/H$ ♀
Rachitogenic	24–27	18	9	6·29	6·07	23·4	22·4
	28–31	35	26	6·49	6·02	21·7	19·9
	32–35	20	15	5·89	5·60	17·4	16·4
	36–39	12	8	4·38	6·07	11·7	16·0
	40–43	10	12	3·16	3·77	7·6	9·1
	44–47	3	8	4·58	1·38	9·7	3·0
	48–51	7	8	1·09	1·09	2·2	2·2
Low Ca	36–39	10	5	0·12	−0·50	0·3	−1·4
	40–43	8	13	−0·43	−0·12	−1·1	−0·2
	44–47	20	12	0·38	−0·24	0·8	−0·5
	48–51	17	8	0·82	0·08	1·6	0·2
	52–55	6	10	1·34	1·55	2·5	2·9
Stock	54–62	20	18	0·17	0·05	0·3	0·1

at both 100 and 150 days of age the humerus and femur of female rats have a higher percentage of ash than those of male rats. The figures given in Table 1 have been analysed in Table 4 according to the sex of the animal. The differences in ash content between the rachitic humerus and femur is shown equally by both sexes.

SUMMARY

1. When low calcification in rats, as measured by the ash content of the humerus, is due to shortage of vitamin D, the femur shows an even lower calcification. When, however, it is due to a deficiency of either Ca or P without a simultaneous shortage of vitamin D in the diet, no difference in calcification between the two bones is observed, neither is a difference found in the bones of rats receiving a stock diet.

2. In the femur both the relatively large metaphysis and the extremely low calcification of this part make the ash content of the whole bone lower than that of the humerus when there is a deficiency of vitamin D in the diet.

3. The differences between the rachitic humerus and femur are not influenced by the sex of the animal.

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The Preparation of Deoxycholic Acid from Cholic Acid

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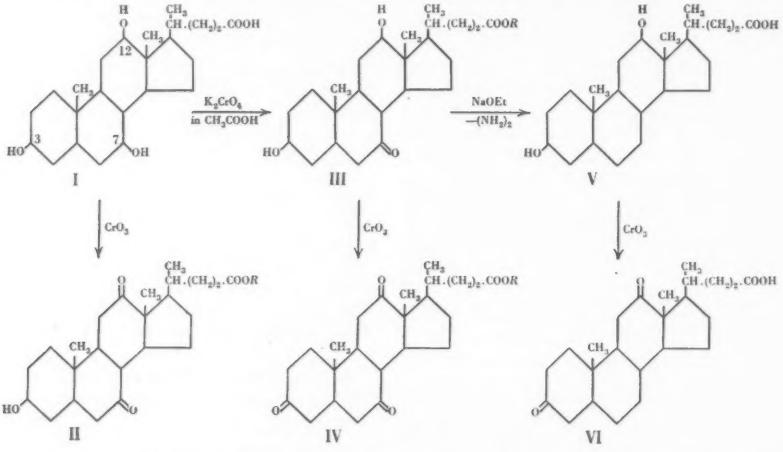
Deoxycholic acid (3:12-dihydroxycholanic acid) has been made from cholic acid in small yield by hydrogenation of the derived 3:12-dihydroxycholenic acid [Boedecker & Volk, 1922], but this method is of academic interest only. The present work, as already stated [Haslewood, 1942], aims at the production of deoxycholic acid by a convenient method from the readily available cholic acid, to supplement the

rather uncertain preparation from bile of Na deoxycholate for bacteriological use. Kaziro & Shimada [1937] showed that cold chromic oxidation of cholic acid (I) gave 3-hydroxy-7:12-diketocholanic acid (II, $R=H$): this was confirmed by Bergström & Haslewood [1939]. Moreover, although Kaziro & Shimada could not obtain a mono-ketonic acid, they indicated that such a substance might be formed, in an overall yield of about 30% of theory. Deoxycholic acid, also of good quality and apparently free from cholic acid, was given in an overall yield of 40–50% of theory by direct reduction of the crude oxidation product. Lithocholic acid may have been an impurity in this final material, for, although it was not apparent therein, it was found in small amount in the mother-liquors. Reduction



since Wieland & Dane [1932] had shown that 7:12-dihydroxycholanic acid was oxidizable to give a 7-keto-12-hydroxy derivative. Direct chromic oxidation of cholic acid has now been made to yield the expected derivatives of 7-keto-3:12-dihydroxycholanic acid (III), easily reduced to deoxycholic acid (V). This, isolated as the ether-complex, was identified by its usual properties and by conversion into the characteristic dehydrodeoxycholic acid (VI).

Controlled chromic oxidation was carried out by the addition of K_2CrO_4 to cholic acid dissolved in acetic acid. Many experiments showed that the highest yield of 7-keto-compounds was obtained (*a*) when the solvent was highly purified acetic acid, and (*b*) when the reaction was greatly slowed. These conditions were met by the addition of measured amounts of K_2CrO_4 in concentrated solution and by buffering the mixture with Na acetate. This method enabled a crystalline semicarbazone (mainly of 7-keto-3:12-dihydroxycholanic acid III, $R=H$) to be isolated in yields of about 45% of theory when chromate was added in amount equivalent to one atom of oxygen. The semicarbazone, however, contained small amounts of cholic acid, which appeared in the final product. This impurity could be removed, but better yields were obtained by using chromate equivalent to two atoms of oxygen, when the semicarbazone, though contaminated with high-melting material, gave a very pure deoxycholic acid [cf. Longwell & Wintersteiner, 1940; Kon & Soper, 1940] was performed with Na ethylate-hydrazine at 200° for 3 hr., with the use of minimal amounts of Na.

The intermediates (III), 7-keto-3:12-dihydroxycholanic acid and its ethyl ester, were obtained from the oxidation product or from the semicarbazone. On acid-alcoholic hydrolysis, the latter gave *ethyl 7-keto-3:12-dihydroxycholanate* (III, $R=C_2H_5$), M.P. (uncorr.) 160–161°, depressed by ethyl 3-hydroxy-7:12-diketocholanate (II, $R=C_2H_5$) but not by ethyl cholate. The same ester M.P. 160–161° was isolated after esterification of the crude chromic oxidation product. It is easier to prepare the 7-keto-ester (the most easily purified of the intermediates) by over-, rather than by under-oxidation of cholic acid, since its separation from ethyl cholate is difficult. The crystalline ester (III, $R=C_2H_5$) was oxidized to ethyl dehydrocholate (IV, $R=C_2H_5$) and reduced, with saponification, to deoxycholic acid. The interesting *7-keto-3:12-dihydroxycholanic acid* (III, $R=H$), from the ethyl ester, separated from dilute aqueous acid as hydrated crystals, M.P. *ca.* 83°, and could be recrystallized from the same solvent although it did not crystallize from organic solvents in the usual way. The cholic acid derivatives could be distinguished from the others here mentioned by the HCl test of Hammarsten [1909], which gave a yellow colour with the 7-keto-compounds (III) and

green colours with mixtures of these and substances with the cholic acid nucleus. The test was of great value during this investigation.

EXPERIMENTAL

All M.P. are uncorrected. Elementary micro-analysis was carried out by Dr H. Nisbet, Edinburgh. Deoxycholic acid was obtained as the ether-complex, M.P. 150–155°. Samples of every preparation obtained in altered conditions were oxidized in the usual way with CrO_3 to give dehydrodeoxycholic acid, crystallizing from dilute alcohol in characteristic glistening leaflets of M.P. 184–186°, decomp., not depressed by authentic material.

Hammarsten's HCl test was done by boiling a mixture of 2–3 mg. of material with 0·2 ml. of strong HCl in a small tube for 1–2 min. The characteristic colours appeared after 1–2 hr.

Preparation of deoxycholic acid. (*a*) *Oxidation and purification.* n g. ($n = 1$–10) of dried and powdered cholic acid were dissolved at 60–70°, in a flask of at least $100n$ ml. capacity, in $10n$ ml. of glacial acetic acid. At the same temperature, $2n$ g. of $CH_3COONa.3H_2O$ crystals were added and dissolved. The mixture was cooled at once to 18–20° and treated with $2n$ ml. ($\equiv 2n$ atoms of O) of K_2CrO_4 solution (31·7 g. of K_2CrO_4/100 ml.), added slowly with shaking, agitation at intervals being continued until all precipitated chromate had dissolved. After at least 24 hr., the solution was diluted with $70n$ ml. of H_2O, and $10n$ g. of crude NaCl were added. When this had dissolved, and after further standing, with occasional shaking, for 24 hr., the liquor was decanted through a filter and the residue and filter washed with H_2O. $10n$ ml. of *N* NaOH followed by $20n$ ml. of H_2O were poured through the filter and back into the original flask. This was now heated, with agitation, until the contents dissolved and the solution gently boiled. After 5–10 min. boiling and when Cr compounds had coagulated, the flask was partially cooled and the still warm mixture treated with $5n$ g. of crude NaCl, dissolved with shaking, followed by $20n$ ml. of *N* H_2SO_4. The precipitate was collected after 16 hr. and well washed on the filter with cold H_2O. It was then allowed to become partially dry, and dissolved in cold alcohol. The filtered solution was evaporated, finally at the pump, leaving 0·8–0·9n g. of a yellowish gum (*product A*) giving a yellow colour in the Hammarsten test.

(*b*) *Reduction.* 5 ml. of an ethyl alcoholic solution containing 40 g./100 ml. of *product A* were added to a metal bomb containing a solution of 0·4 g. Na in 5 ml. ethyl alcohol, with 2 ml. of hydrazine hydrate (95/98%). The mixture was carefully heated almost to boiling; then the bomb was sealed and heated at 200–210° for 3 hr. An aqueous extract (*ca.* 200 ml.) of the contents of the cooled bomb was acidified with dilute H_2SO_4, the precipitate being warmed gently to disperse gels. After 2–3 hr., the white solid was collected, washed with water and dissolved in cold alcohol. Evaporation of the filtered solution left a gum which crystallized at once when about 10 ml. of ether were added. After warming with the ether, the product was allowed to stand for several hours with occasional shaking, and was finally cooled to 0°. The crystals were collected (suction), washed with ice-cold ether, and dried. Yield, 1·1–1·2 g.; small white needles M.P. 150–155°, HCl-test, faint yellow-green. 0·2 g. of this substance, from 2–3 ml. of acetic acid, gave 0·17 g. of white needles M.P. 139–141°.

4 g. of residue obtained on evaporation of the ether mother liquors from (*b*) were dissolved in a little fresh ether. During 24 hr., crystals accumulated. The liquor was decanted and the crystals washed with ether and cold alcohol, collected, and dried. This material (0·15 g.) had M.P. 184–186°, not depressed by lithocholic acid; it was not obviously affected by HCl.

Semicarbazone. To *product A*, from n g. of cholic acid, were added $n/2$ g. of semicarbazide hydrochloride, $n/2$ g. of Na acetate crystals, and $5n$ ml. of 50% (by vol.) alcohol/water. This mixture was warmed till all had dissolved, boiled under reflux for 1 hr. and allowed to stand 3–5 days. The solid was collected (suction), washed with 50% alcohol, water, alcohol, and ether, and dried at 60–80°. Yield, 0·6n g., M.P. >330°; HCl-test, yellow. (Found, N, 11·2%.) When the oxidation (*a*) was done with $1{\cdot}1n$ ml. of K_2CrO_4 solution ($\equiv 1{\cdot}1n$ atoms of O), the semicarbazone (0·5n g.), prepared as described, formed groups of white needles, M.P. 250° (decomp.), which gave a blue HCl-test as also did the deoxycholic acid and 7-keto-derivatives from them. (Found, N, 8·6%.) 0·5 g. of this latter product, after boiling with 5 ml. of alcohol under reflux for 30 min. left 0·32 g. of undissolved crystals, M.P. 265°, decomp.; HCl-test, yellow-green. (Found, N, 10·0%. $C_{25}H_{41}O_5N_3$ requires N, 9·1%.) A product of M.P. 267°, decomp.; HCl-test, green, was given when $K_2CrO_4 \equiv 1{\cdot}5n$ atoms of O was used for the oxidation (*a*).

The above semicarbazones could be reduced by heating (in a sealed bomb at 200–210° for 3 hr.) 1 g. samples with a solution of 0·25 g. of Na in 10 ml. ethyl alcohol, with 1 ml. of hydrazine hydrate. The deoxycholic acid, obtained as under (*b*), had similar properties, that from the semicarbazone M.P. >330° appearing to be of especially good quality. Overall yield of deoxycholic acid via the semicarbazone, about 30% of theory.

Ethyl 7-keto-3:12-dihydroxycholanate. (i) 6·5 g. of *product A* were boiled for 45 min. under reflux with 13 ml. of a mixture of ethyl alcohol (20 ml.) and concentrated H_2SO_4 (5 ml.). The cooled solution was diluted with water and extracted three times with ether. The ether, washed with water and Na_2CO_3 solution, was evaporated, and the residue crystallized from methyl alcohol; 1·6 g. of crude crystals were obtained.

(ii) 3 g. of semicarbazone M.P. >330° were boiled under reflux for 1 hr. with 30 ml. of a mixture of ethyl alcohol (40 ml.), H_2O (10 ml.), and conc. H_2SO_4 (5 ml.). From the cooled and diluted solution, crude crystals (0·9 g.) were obtained exactly as under (i). The ester crystallized from methyl alcohol in large colourless rectangular plates or prisms, which, after drying at 70–80°, had M.P. 160–161°, not depressed by ethyl cholate. (Found, C, 72·0; H, 9·9%. $C_{26}H_{42}O_5$ requires C, 71·9; H, 9·7%.) The compound formed glistening leaflets from a diluted methyl alcoholic solution: it gave a yellow colour with strong HCl.

A purified sample of ethyl 7-keto-3:12-dihydroxycholanate was oxidized in the usual way with CrO_3 to ethyl dehydrocholate, M.P. 222–223° (decomp.), and another sample was reduced as under 'Semicarbazone' to deoxycholic acid (0·13 g. from 0·2 g. of ester, M.P. 160–161°). By the same process, 0·2 g. of ethyl cholate gave 0·14 g. of cholic acid.

7-keto-3:12-dihydroxycholanic acid. 0·3 g. of the above ester, M.P. 160–161°, was boiled under reflux for 30 min. with a mixture of Na (70 mg.), ethyl alcohol (1·5 ml.), and H_2O (1 drop), the ester being added after the Na had all dissolved. After dilution the cold solution was filtered, further diluted to 200 ml., heated to boiling and acidified with

5 ml. of *N* H_2SO_4. After 3–4 days, the white needles which had formed were collected and dried *in vacuo*. Yield, 0·13 g., M.P. 83°, giving a yellow colour with concentrated HCl and in the Liebermann-Burchard test, and a red colour with concentrated H_2SO_4. There was difficulty in removing solvent from this substance for analysis. (Found: C, 70·8; H, 9·5%. M (titration), 395. $C_{24}H_{38}O_5$ requires C, 70·9; H, 9·4%; M, 406.) The acid could be recrystallized by acidification of a hot alkaline solution as described, but did not form crystals from organic solvents. It dissolved easily in alcohol, acetone, and acetic acid, and to some extent in ether, but appeared to be insoluble in benzene and light petroleum.

SUMMARY

1. By a simple three-stage process (oxidation, purification, reduction), cholic acid has been converted into deoxycholic acid, with an overall yield of 40–50% of theoretical.

2. Preferential oxidation of cholic acid at C_7 was performed by adding measured amounts of concentrated K_2CrO_4 solution to cholic acid dissolved in acetic acid buffered with Na acetate.

3. The partially purified product was then reduced with Na ethylate-hydrazine, yielding deoxycholic acid. Deoxycholic acid was also prepared from an impure semicarbazone, the preparation and properties of which were used to follow and define the conditions of the oxidation. The intermediate 7-keto-3:12-dihydroxycholanic acid and its ethyl ester have been isolated and examined.

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Dental Depigmentation in the Rat

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In normal rats the anterior surfaces of the incisor teeth are a rich brown or orange. According to Marshall [1921] the pigment is confined to the outer fibrous layer. The whitening of the teeth which occurs in vitamin A deficiency has been described by Wolbach & Howe [1925; 1933], Smith & Lantz [1933], Schour, Smith & Hoffman [1938], Schour, Hoffman & Smith [1941], Irving & Richards [1939] and H. Mellanby [1939]. In recent experiments on the interaction of vitamin A and vitamin E, Davies & Moore [1941] found that under certain circumstances whitening of the teeth may result from the exclusion of vitamin E from the diet. It was suggested that this lesion might be caused by deficiency of either vitamin. Evidence in favour of this view is given in the present communication.

EXPERIMENTAL

Grading of depigmentation. To assess the degree of depigmentation in individual rats each tooth was awarded marks according to the following arbitrary scale.

Description	Marks given
White	25
Faint brown mottling on white	20
Cream	15
Mottled brown and white	10
Deep cream	10
Light brown	5
Brown	0

Thus the dental depigmentation of a rat with four completely white teeth is expressed by 100 marks, while four completely brown teeth are indicated by 0 marks.

The cure of white teeth in rats deficient in vitamin A by the administration of carotene

In routine tests for vitamin A in samples of butter and margarine, male albino and piebald rats were given a basal diet of caseinogen (Glaxo, alcohol extracted) 20%, sugar 60%, arachis oil 15%, salt mixture 5% and dried yeast 10%. Vitamin E was supplied as 1 mg. weekly of *dl*-α-tocopherol acetate dissolved in arachis oil, and vitamin D as 1 drop of 'Radiostol' (B.D.H.). In addition small doses of

the butter or margarine were given to supply enough vitamin A to allow fairly rapid growth. At the end of the test 12 rats with completely white teeth were nevertheless available for use in the present investigation. These were kept on the same basal diet and divided into 4 groups, which were dosed with a solution of carotene in arachis oil at levels of 2, 6, 16 and 44 i.u. per rat daily.

The changes in weight during the next 59 days are shown in Table 1.

Table 1. *Growth of rats receiving different doses of carotene*

Daily dose of carotene i.u./day	Group 1			Group 2			Group 3			Group 4		
	2			6			16			44		
Rat no.	1	2	3	4	5	6	7	8	9	10	11	12
Wt. (g.) on day 1	178	173	210	160	154	143	166	148	182	126	112	187
Wt. (g.) on day 60	174	130	Died	210	197	180	219	220	222	194	193	267
Mean increase in wt. (g.)		−23			+43			+55			+76	

Signs of the return of brown colour to the teeth were seen in some rats of groups 3 and 4 after 30 days, and after 60 days most of the animals had almost normal teeth. The teeth in groups 1 and 2 remained almost completely white. The marks given are shown in Table 2.

Table 2. *Dental depigmentation in rats receiving different doses of carotene*

Daily dose of carotene i.u./day		Group 1			Group 2			Group 3			Group 4		
		2			6			16			44		
Rat no.		1	2	3	4	5	6	7	8	9	10	11	12
Depigmentation of teeth (arbitrary marks)	Day 1	100	100	100	100	100	100	100	100	100	100	100	100
	Day 60	75	90	Dead	90	90	80	20	20	10	10	10	45
Mean mark at end of exp.			83			87			17			22	

Examination of the unerupted portions of the teeth gave similar results.

The cure of white teeth in rats deficient in vitamin E by the administration of dl-α-*tocopherol*

Female albino rats were kept for periods of 4 or 7 months on a diet deficient in vitamin E, consisting of caseinogen ('light white') 25%, sugar 50%, lard 10%, dried yeast 10% and salt mixture 5%. Vitamin A was supplied at the level of about 1000 i.u./week in the form of halibut liver oil. The animals, most of which showed marked dental depigmentation, were then divided into two groups, each comprising three rats which had received the deficient diet for 4 months, and four rats which had received the same diet for 7 months. One group was now given 1 mg. of tocopherol acetate weekly, the other received no vitamin E. In connexion with another experiment each group received occasional injections of lard, a treatment which would not be expected to influence the present results.

The weights of the rats at the time of division into groups, when they were already nearly full grown, and after further periods of 84 and 203 days, are given in Table 3.

In group 1 two rats died after receiving tocopherol for about 3 months. With rat no. 8 death was probably due to sepsis following the injection of lard. Rat no. 7 was not autopsied, but it was emaciated and had overgrown teeth which may have caused difficulty in eating. The data in Table 3

Table 3. *Weights of rats receiving vitamin E-deficient diet*

Group 1. Given 1 mg. of tocopherol acetate per rat, each week

Rat no.	13	14	15	16	17	18	19
Wt. (g.) on day 1	202	250	196	187	169	182	186
Wt. (g.) on day 84	227	227	214	245	192	203	171
Wt. (g.) on day 203	237	239	237	Dead	203	217	Dead

Mean increase of the five rats surviving 203 days = 27 g.

Group 2. Given no added tocopherol

Rat no.	20	21	22	23	24	25	26
Wt. (g.) on day 1	165	176	182	202	165	167	192
Wt. (g.) on day 84	187	192	197	211	177	185	197
Wt. (g.) on day 203	170	192	188	204	173	177	182

Mean increase of five rats showing greatest growth = 9 g.

show that little change in weight occurred in either group.

The degree of dental pigmentation at the beginning of the test period was less uniform in both groups than in the preceding experiment in which deficiency of vitamin A had been involved. The individual marks after various periods are shown in Table 4.

The mineral content of the teeth in avitaminosis E

Estimations of ash content and of fluorine in white and normal brown teeth were kindly made by Dr H. H. Green. The teeth examined were taken from rats which had been used in previous experiments [Davies & Moore, 1941]. The results are given in Table 5. There would therefore appear to be a marked increase of the ash content in whitened teeth. The values for F were within the normal range in both groups.

Table 4. *Dental depigmentation of rats receiving vitamin E-deficient diet*

Group 1. Given 1 mg. tocopherol acetate per rat, each week

	Rat no. ...	13	14	15	16	17	18	19	Mean
Depigmentation of teeth (arbitrary marks)	Day 1	100	0	100	70	70	100	100	76
	Day 84	0	0	0	0	0	0	100	15
	Day 203	0	0	0	Dead	0	0	Dead*	0

Group 2. Given no added tocopherol

	Rat no. ...	20	21	22	23	24	25	26	Mean
Depigmentation of teeth (arbitrary marks)	Day 1	100	90	100	90	90	90	90	93
	Day 84	100	70	90	60	70	70	65	75
	Day 203	100	50	90	90	95	90	95	87

* Teeth still white at death, but malformed and overgrown.

If we omit from consideration rat no. 14, which had brown teeth at the beginning, and also no. 19, which was diseased, the remaining five rats in group 1 all showed complete cure as the result of treatment with tocopherol. Of the seven rats in group 2 only no. 21 showed slight improvement, the teeth of the other six remained almost completely white.

At the end of the experiment the five surviving rats of group 1, and five rats of group 2, were killed and the vitamin A reserves in their livers estimated by the $SbCl_3$ method. For group 1 the mean total reserve was 14,000 i.u. For group 2, in confirmation of previous observations on the effect of vitamin E deficiency in lowering the vitamin A reserve [Moore, 1940], the mean value was only 4700 i.u., which, however, is still about twice the reserve found in a wild rat shot near this laboratory [Davies & Moore, 1934].

Table 5. *Mineral analysis of teeth from normal rats and from rats deficient in vitamin E*

Rat no.	Wt. of lower incisors g.	Dry matter %	Ash content %	Fluorine p.p.m.* original tooth
	(*a*) Normal rats (brown teeth)			
1	0·198	86·5	64·5	50
2	0·129	87·5	64·0	115
3	0·107	89·5	65·0	140
4	0·140	89·5	62·5	140
Mean	0·144	88·2	64·0	111
	(*b*) Deficient rats (white teeth)			
1	0·141	89·0	67·5	35
2	0·189	90·5	71·5	80
3	0·166	89·5	66·5	180
4	0·125	90·5	72·0	80
Mean	0·155	89·9	69·4	94

* p.p.m. = parts per million.

DISCUSSION

The above experiments suggest that whitening of the teeth in the rat results from deficiency of either vitamin A or of vitamin E, and may be cured by the restoration of whichever of these vitamins is missing. Since vitamin E influences the efficiency of storage and utilization of vitamin A [Moore, 1940; Bacharach, 1940; Davies & Moore, 1941] the occurrence of a common lesion in deficiency may imply a further aspect of their interaction. Although deficiency of vitamin E may under some circumstances cause a secondary deficiency of vitamin A, indicated by the premature depletion of the vitamin A reserves in the liver, whitening of the teeth in vitamin E deficiency may occur even when large amounts of vitamin A are given and the reserves in the liver are high. While therefore the dental lesion may possibly result from a disturbance in the function of vitamin A as a result of the absence of vitamin E, a material deficiency of vitamin A is not an essential aetiological factor.

Dental depigmentation also occurs in fluorosis [McCollum, Simmonds, Becker & Bunting, 1925] and in Mg deficiency [Duckworth, 1942]. The lesion may therefore result from various dietary abnormalities, which may or may not be interrelated. Moreover, Irving [1942] has detected lesions in the enamel-forming organs of rats deficient in vitamin E, distinct from those observed in avitaminosis A, at a stage of the deficiency when the

teeth are still brown. Depigmentation may therefore be the final outcome of different initial histological lesions.

The persistence of dental depigmentation in rats which have been deprived of vitamin A and subsequently treated with small doses of this vitamin affords an excellent demonstration of the reality of the conception of suboptimal nutrition. Rats in the group given 6 i.u. of vitamin A daily grew almost as rapidly as those given 16 i.u., but whereas 16 i.u. was sufficient to restore the brown colour of the teeth, 6 i.u. was inadequate. Ability to sustain rapid growth does not therefore necessarily imply that the animal is receiving the full supply of nutrients necessary for perfect health.

SUMMARY

1. Whitening of the teeth in rats was induced by deficiency of either vitamin A or vitamin E, and could be cured by restoration of whichever vitamin was missing.

2. A low dose of vitamin A, which was sufficient to restore rapid growth in rats previously deprived of this vitamin, was ineffective in restoring the normal brown colour of the teeth.

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The Microbiological Degradation of Steroids

1. THE STEROL CONTENT OF SOILS

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The wide distribution of free or esterified sterols in animal and vegetable tissues must result, during the decay of such organic material, in the deposition in soils of an appreciable quantity of various sterol substances. Schreiner & Shorey [1909; 1911] have reported the isolation from soils of two sterols. The first of these, agrosterol, was present in the free condition, and was considered to be the sterol component of some plant tissue, or to have originated by structural alteration from some substance of entirely different composition present in the soil. The second, phytosterol, M.P. 135°, occurred only in the form of its esters, which were regarded as plant residues remaining unchanged in the particular peaty soil under examination. In general, however, the sterol content of soils seems to have received but little attention. The investigation of the various factors influencing the accumulation of sterols in, and their disappearance from, different types of soil forms the basis of the present work.

METHODS

The quantitative determination of soil sterols is attended by considerable difficulty. Humus material may be present in proportions of from less than 1% in the largely mineral soils to almost 100% in pure peat, and the purification of the sterols thus becomes lengthy and tedious. It has been found that a preliminary solvent treatment of the soil, followed by purification of the crude sterol extracts by means of the insoluble digitonides, yields solutions sufficiently pure for colorimetric estimation.

The soil sample is thoroughly dried in an air oven at 80°, and, after being ground in a mortar, is passed through a 30-mesh sieve to remove pebbles, plant fragments, etc. 1 kg. of soil obtained in this way is extracted continuously with acetone. After removal of the solvent by distillation, the brownish gummy residue is refluxed with a slight excess of alcoholic KOH for 30 min. The solvent is removed on a water bath, and the dark-coloured viscous liquid remaining taken up in a large excess of anhyd. K_2SO_4. This solid is

extracted for 4 hr. in a Soxhlet apparatus with petroleum, B.P. 40–50°, and the extract made up to known volume. The determination of sterol in this solution is based upon the method of Kelsey [1939]. A portion containing approximately 0·5 mg. sterol is evaporated to 0·5 ml. in a 15 ml. graduated centrifuge tube. 4·0 ml. of a 0·2% solution of digitonin in 95% alcohol are added, and the mixture evaporated to dryness. The solid residue is boiled with 12 ml. petroleum, B.P. 40–50°, the tube and contents centrifuged, and the supernatant yellowish liquid discarded. This is repeated until the extracts are no longer coloured. The residual solid is then gently refluxed with 7·0 ml. benzene for 45 min. to decompose the digitonide, and after being concentrated to 3 ml., the contents of the tube are finally diluted to 12 ml. with petroleum, B.P. 40–50°. Digitonin separates on thorough stirring, and is centrifuged off. The supernatant liquid is decanted, and the residue re-extracted three times with light petroleum. The combined extracts are evaporated to dryness, and the residue taken up in 5·0 ml. $CHCl_3$. This solution is transferred to a 10 ml. glass-stoppered cylinder. 0·5 mg. cholesterol dissolved in 5·0 ml. $CHCl_3$ is measured into a second cylinder, and 1·0 ml. acetic anhydride and 0·1 ml. conc. H_2SO_4 are added to each. The solutions are thoroughly mixed, and are allowed to stand in a bath at 24° for 15 min.; the colours which have developed are then compared in a colorimeter.

Neither the digitonin precipitation used as the final purification in the above method, nor the Liebermann-Burchard colour reaction, is itself specific for sterols. Digitonin precipitation in the steroid group appears [Windaus, 1909] to depend upon a free —OH at C_3 in the cholane nucleus, and the *cis*-position of this —OH to the —Me at C_{10}. Thus, insoluble digitonides are also given by several bile acids, certain cholane cardiac poisons and their aglycones, and by various other compounds. The cardiac glycosides, in common with the saponins, many bile acids and other substances, also give the Liebermann-Burchard reaction for sterols. During the various purification stages described these compounds are eliminated, with the possible exception of certain cardiac aglycones which give both digitonin precipitates and positive colour reactions. These compounds, however, are probably far rarer in soils than the sterols, and no evidence of their presence in any of the final solutions has been obtained with the Legal nitroprusside test.

The H_2SO_4-acetic anhydride colour may thus be regarded as indicative of the total sterol content of the soil, irrespective of whether the sterol was originally present in the free state, as its fatty acid esters, or in the form of its glycosides. No attempt has been made to identify individual sterols, the result in all cases being expressed in terms of cholesterol.

The accuracy of the method has been tested by determining on a number of soils the percentage recovery of cholesterol added to dried, sieved soil.

Table 1. *Recovery of added cholesterol*

Initial sterol content mg./kg. soil	Cholesterol added mg.	Total sterols by analysis mg./kg. soil	Cholesterol recovered mg.	Recovery %
1·8	61·3	61·5	59·7	97·4
0·9	52·4	54·7	53·8	102·7
20·1	25·7	45·2	25·1	97·7
Nil	18·9	18·1	18·1	95·7

The difference in the figures obtained in four experiments with the original soil and that containing added cholesterol indicates the recovery of cholesterol (Table 1). The recoveries have been of a consistently high order.

DISCUSSION

Distribution of sterols in British soils

The soils examined in this survey have been collected from widely distributed areas throughout the British Isles, and include the majority of soil types found in this region. They have, in general, been taken from a depth of from 2 to 6 in., after removal of the surface layer of living or dead vegetation, but in certain instances where the profile showed deep peaty humus layers, sampling has been carried out at a depth of several feet.

Table 2. *Sterol contents of British soils*

Vegetative type	Source	Soil type	No. of samples	Sterol substances present mg./kg. soil
Forest land	Oak and oak-birch associations (Sherwood Forest)	Coarse acid sand	6	0·6– 1·4
	Oak (Weald)	Light loam to heavy clay	2	0·1, 0·8
	Beech (Sherwood Forest, Epping Forest, Chiltern plateau)	Coarse acid sand	5	0·2– 1·2
	Beech (South Downs)	Shallow, alkaline, light loam	2	0·8, 2·5
	Beech (Chiltern escarpment)	Strongly alkaline marl	1	3·2
Grass land	Acid grassland (southern Pennines, Malvern Hills)	Acid sand or loam	2	Nil, 0·8
	Neutral grassland (Trent and Thames valleys)	Chiefly loams	4	0·4– 1·5
	Basic grassland (Chiltern escarpment)	Shallow, alkaline, marls	3	0·6– 2·7
Fen land	Wicken Fen, Cambridge, Norfolk Broads	Black, slightly alkaline peat	4	2·3– 8·5
Bog land	Peat moors, Athlone, Spring moors, New Forest, Dartmoor	Deep, acid peat	5	7·9–12·7
Heath land	Sherwood Forest, Pennine moors, Yorkshire moors, Central Scottish highlands	Coarse sand	6	Nil– 1·1
Arable land	Various	Various	12	Nil– 6·3

The results (Table 2) show clearly that definite relationships exist between sterol content and soil type. Deposition of sterols must depend primarily upon the nature and number of the animal and plant inhabitants, so that deposition will be at a maximum in soils favourable to the maintenance of a flourishing population. A characteristic of such soils, however, is the rapidity with which humus material undergoes disintegration. Since the sterol content of these soils shows no continued increase, this component of the humus is presumably decomposed or disposed of in some other manner. In soils of the opposite type there is a marked accumulation of sterols. Extreme acidity, lack of aeration, and excessive water content are all factors which, either individually or collectively, inhibit the decomposition of humus and its constituent sterols, and are thus directly responsible for the high sterol contents of bog and fen peats. In arable soils these adverse factors have been counteracted, and, whilst it is found that sterols are present in varying amount, the content of any particular soil shows a gradual decrease unless replenished by organic manuring.

Agencies responsible for sterol disappearance

In view of the insolubility of the sterols, and of their association with natural fats and oils, their disappearance from soils cannot be attributed to the leaching action of rain-water, but is probably the result of degradation of the sterol molecule by the co-ordinated activities of a variety of soil micro-organisms. Evidence in favour of this theory has been obtained experimentally in the following manner.

Six 0·05 g. quantities of cholesterol were accurately weighed, and each intimately mixed with 25 g. soil. The mixtures were placed in separate porous flower pots in which the drainage holes had been closed with watch-glasses, and the pots buried with their tops 1 in. below the surface of a well-worked loamy garden soil. One group of three pots was watered weekly with rainwater, whilst the second group was treated with a dilute solution of $HgCl_2$.

Table 3. *Rate of disappearance of sterols from soil under different conditions*

Time months	Sterol content (mg.) Group I (water)	Group II ($HgCl_2$)
0*	50·0	50·0
3	42·7	49·2
6	29·6	49·8
12	18·3	48·9

* The sterol content of the untreated 25 g. sample of soil is negligible.

At intervals of 3, 6 and 12 months respectively, one pot from each group was removed, and the sterol content of the soil determined (Table 3). In the first group sterol disappeared, but the sterilizing action of the $HgCl_2$ inhibited microbiological activity in the second group so that the sterol content altered little. Similar experiments, using 'organic' cholesterol in the form of brain tissue and egg yolk, gave comparable results although disappearance of sterol was here less rapid than with the crystalline material.

The nature of the biological agents responsible for sterol breakdown is being investigated, and there are indications that decomposition is initiated by certain acid-tolerant, aerobic bacteria.

SUMMARY

1. A method is described for the determination of soil sterols.

2. The sterol content of a wide range of British soils has been determined.

3. Accumulation of sterols in soils occurs under conditions of extreme acidity, waterlogging and lack of aeration.

4. Where these conditions are reversed, as in arable soils, the sterol content gradually decreases.

5. The disappearance of soil sterols has been demonstrated experimentally, and has been shown to result from the activities of microbiological agents.

The author wishes to express his thanks to colleagues who have assisted in obtaining soil samples.

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Liver Catalase

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Stern [1935–6] isolated biliverdin (dehydrobilirubin) from the split products of horse liver catalase; the pigment was identified by Lemberg [cf. Lemberg & Wyndham, 1936–7]. Biliverdin was not present in the catalase preparation as such, but as a bile pigment haem compound bound to a protein similar to or identical with that characteristic of catalase [Lemberg & Wyndham, 1936–7]. After reduction

and transformation to pyridine-haemochromogen it gave the absorption spectrum of verdohaemochromogen. At that time it was assumed that the bile pigment haemoprotein was an impurity.

Sumner & Dounce [1937] obtained ox liver catalase in crystalline form. After repeated recrystallization fission with HCl in acetone of the enzyme yielded haemin and a blue compound, which was believed to contain iron and to be different from both biliverdin and verdohaemochromogen. While one-half of the catalase iron was firmly bound as protohaemin, the other half ('blue iron') was easily ionized by treatment with HCl, but unlike the iron of ferritin, which sometimes accompanies catalase, it was not precipitated with the protein. Iron of bile pigment haem compounds is readily set free by acid.

Lemberg, Norrie & Legge [1939] reported the isolation of crystalline biliverdin hydrochloride from crystalline ox and horse liver catalases. In view of the constant molar ratio of biliverdin to haemin (about 1 to 3), it was then assumed that the bile pigment haem formed an essential part of the catalase molecule. Another possibility, however, was mentioned, i.e. that the protohaem group in catalase may be oxidized to bile pigment haem by H_2O_2, leaving the protein component unaltered. At that time we were unable to find experimental support for this assumption.

Stern [1940] accepted this latter explanation of the presence of bile pigment haem in crystalline catalase as the correct one, while Agner [1939] and Theorell [1940] claimed that iron-free biliverdin was admixed with catalase. Agner based this claim on the isolation of a highly active horse liver catalase (of Kat.-f. 62,000),* which contained 0·086 % protohaem iron (83 % of the total iron in the form of protohaem and only 10 % in the form of 'blue iron'), but still gave biliverdin on splitting with acid. No quantitative estimations of the bile pigment were carried out, however, nor was any explanation given for the lack of success in separating catalase and biliverdin by fractional precipitation with $(NH_4)_2SO_4$, crystallization and cataphoresis.

Sumner, Dounce & Frampton [1940] obtained horse liver catalases in which the content of the iron accompanying biliverdin was inversely proportional to the specific activity. This indicated that the bile pigment haemoprotein is inactive and present in catalase in varying proportion.

We arrived at the same conclusions independently and with other methods. While Sumner studied the ionizable iron fraction, we investigated the molar ratio of bile pigment to haemin obtained by splitting catalase with acids, and the absorption spectra of the CO-haemochromogens obtained from catalase.

We have also shown that the bile pigment haem content of catalase can be increased by treatment with H_2O_2 in the presence of ascorbic acid, and that ascorbic acid inhibits catalase by accelerating its destruction by H_2O_2.

EXPERIMENTAL

Isolation and purification of catalase

Crystalline ox liver catalase was readily prepared by the method of Sumner & Dounce [1937]. The crystals can be further purified by washing with distilled water, and are dissolved by dialysing the suspension in distilled water against $M/50$ phosphate buffer pH 7·8; they are then recrystallized according to the method of Sumner & Dounce.

The crystals have the form of fine needles or small platelets described by Sumner, but on long standing in the refrigerator strong solutions of ox liver catalase occasionally deposit very large thick hexagonal plates which are no longer easily soluble in phosphate buffer.

Horse liver catalase. The dioxan method of Sumner *et al.* [1940] was not consistently successful in our hands, and we used the earlier stages of the method of Zeile & Hellström [1930]. The difficulty due to phosphate in the eluates [Agner, 1938] is easily overcome by removal of the phosphate by thorough dialysis at 0° against distilled water. After this the fractionation of catalase and ferritin even in strong solutions offers no difficulties. Crystallization and recrystallization were carried out according to Sumner *et al.* [1940]. Horse liver catalase crystals are more readily soluble in water than ox liver catalase.

Amorphous horse liver catalase was prepared by the methods of Zeile & Hellström [1930] and of Keilin & Hartree [1936].

Methods

Total protein was estimated with a micromodification of the method of Bick [1936].

Catalase activity. The activity was determined by the method of Zeile & Hellström [1930]. We can confirm that the values of $-(\Delta \log C_{H_2O_2})/\Delta t$ show a noticeable decrease with time even under their conditions. Sumner extrapolates these values for $t=0$, but does not mention his method of extrapolation. By a different calculation one can determine the exact value of k_0.

* Kat.-f. (Katalasefähigheit) was used by Euler to designate the activity of a solution containing 1 g. of catalase in 50 ml. We have previously used the translation 'catalase factor' in order to avoid the grammatical difficulties connected with the use of Kat.-f. A better translation is specific catalase activity (Sp.C.A.) and this will be used in this paper.

According to Nosaka [1927] and Maximowitsch & Awtonomova [1928] the kinetics of destruction of H_2O_2 by blood catalase are given by the equations $-dC = k\,EC\,dt$ and $-dE = k'\,EC\,dt$, where C is the concentration of H_2O_2,

E that of catalase, k the reaction constant of the destruction of H_2O_2 by the enzyme and k' that of the destruction of the enzyme by H_2O_2. Integration yields the equation

$$\ln \frac{C_0}{C} - \ln \frac{C_0+A}{C+A} = k' t A, \qquad (1)$$

where A is an integration constant defined as

$$A = \frac{k E_0}{k'} - C_0. \qquad (2)$$

If the H_2O_2 concentrations C_0, C_1, C_2 and C_3 at the times t_0, t_1, t_2 and t_3, where $t_3 - t_2$ equals $t_2 - t_1$, are known, kE_0, the required value for the activity at $t=0$, can be calculated. The simplest way of finding A is by approximation from the equation

$$\ln \frac{C_1}{C_2} - \ln \frac{C_2}{C_3} = \ln \frac{C_1+A}{C_2+A} - \ln \frac{C_2+A}{C_3+A}. \qquad (3)$$

Inserting the value of A into equation (2) yields k' and from equation (1) kE_0 is then found as $(A+C_0)k'$. We have followed, however, the customary procedure of using $\log_{10}$ for calculation of the catalase reaction constant.

Table 1 shows that the course of H_2O_2 destruction by liver catalase can be satisfactorily calculated by assuming the validity of these equations, and that the action of liver catalase, therefore, obeys the same kinetic laws as that of blood catalase.

Table 1. *Kinetics of H_2O_2 destruction by liver catalase*

$A=12$, $k'=0{\cdot}0045$

t min.	$C_{H_2O_2}$ found as average of 2 estimations	$C_{H_2O_2}$ according to theory
0	9·03	9·03
0·5	8·11	8·07
1·5	6·62	6·64
3·5	4·59	4·55
4·5	3·86	3·85
6·5	2·81	2·75
7·5	2·41	2·35
9·5	1·80	1·75
10·5	1·56	1·53
20·0	0·43	0·45

In several instances we have observed a slight fall of A with time which would indicate that the speed of destruction of the enzyme does not decrease as much as the H_2O_2 concentration. The curvature of the graph of $C_{H_2O_2}$ plotted against time is slightly less than expected from theory.

A simpler calculation of k_0 can be derived from the equations: $-dC = kCE\,dt$ and $-dE = k'E$. It follows

$$\ln C_0 - \ln C = \frac{k}{k'} E_0 (1 - e^{-k't}). \qquad (4)$$

The destruction of the enzyme does, of course, depend on the H_2O_2 concentration, and the curvature of the $C_{H_2O_2}$ graph is greater than would be expected from equation (4). Under the conditions of the estimations, however, with a low enzyme concentration and a small fall of H_2O_2 concentration, the equation

$$k_0 = \text{antilog.} \left\{ \frac{\frac{1}{2}(t_1+t_2)\log k_{t_1-t_2} - \frac{1}{2}(t_2+t_3)\log k_{t_2-t_3}}{\frac{1}{2}(t_3-t_1)} \right\}, \qquad (5)$$

which is derived from equation (4) yields results which agree with those found by the formulae (1)–(3) within the experimental error.

In order to compare our results with those of Sumner, we have calculated k_0 from the examples given by Sumner & Dounce [1939, p. 442] with our formulae. The values given by these authors are 0·0510 and, with half the enzyme concentration, 0·0247. With the formulae (1)–(3) we find 0·0511 and 0·0254, with formulae (5) 0·0505 and 0·0248. Our results are, therefore, strictly comparable with those of Sumner. Sumner & Dounce do not mention the volume of diluted catalase solution added to 50 ml. of H_2O_2, but if this was 0·5 ml., as used by Zeile, the specific activities were 24,000 and 23,200, and not 35,000 and 34,500 as given by them.

Protohaem in catalase was determined spectrophotometrically by measurement of the absorption at 558 mμ of the haemochromogen formed on adding NaOH, final conc. 1·25%, followed by a few mg. $Na_2S_2O_4$. We have found no difference in the height of the absorption band on the addition of pyridine, nor any cloudiness of the solution, except with partly denatured catalase. The concentration of protohaemin in mg./ml. is $(\epsilon_{555\,m\mu} \times 10^3)/47{\cdot}5l$, where l is the length of layer through which the light passes, and 47·5 the extinction coefficient of a solution of protohaemochromogen containing 1 mg. haemin/ml.

Estimation of bile pigment content and bile pigment:haemin ratio. The isolation of bile pigments from catalase treated with acetic acid was by the method of Lemberg, Lockwood & Legge [1941*b*]. About 5 ml. of catalase solution of $k=5000$ sufficed. No ascorbic acid was added, since acidified catalase does not oxidize biliverdin. The 66% acetic acid solution was diluted with the same volume of peroxide-free ether, and the almost colourless protein removed by washing with Na acetate solution and water. These washings were saturated with $(NH_4)_2SO_4$, and re-extracted with ether [cf. Lemberg *et al.* 1941*b*, p. 369]. The bile pigments were extracted by repeated shaking with 20% HCl.

The isolation of crystalline biliverdin hydrochloride from crystalline liver catalase has been described [Lemberg *et al.* 1939]. The crystals possessed the typical form and optical and solubility properties, and on oxidation of the complex zinc salt with iodine [cf. Lemberg, 1935] the characteristic fluorescence and absorption band of the biliviolin zinc complex salt were obtained.

In our first estimations we determined biliverdin in the acid extracts colorimetrically, by comparison with a biliverdin standard prepared from pure crystalline biliverdin. Later it was recognized that the extracts contain small amounts of biliviolin (usually about 20% of total bile pigment). The values for bile pigment content and for the molar ratio (bile pigment Fe)/(bile pigment Fe + protohaem Fe), obtained by this method, are therefore too small, but can be compared among themselves. The results obtained by this method are given in Table 2.

In the later experiments, summarized in Table 3, biliverdin and biliviolin were measured spectrophotometrically according to method III [Lemberg *et al.* 1941*b*].

After extraction of the bile pigments the ethereal solution of haemin was washed once with water, and protohaem was then extracted with NH_3 and determined spectrophotometrically as pyridine-haemochromogen [Lemberg, Legge & Lockwood, 1941*a*]. Usually 50–70 % of the total protohaem of catalase was thus recovered. The remainder clings to the denatured protein. We shall give below evidence showing that the bile pigment in catalase is present in the form of a bile pigment haem compound, from which bile pigment is split off quantitatively. The ratio (bile pigment Fe)/(bile pigment Fe + protohaem Fe), the latter being called 'total haem Fe', given in Tables 2 and 3, was calculated assuming that the recovery of bile pigment was as complete as that of protohaem.

Bile pigment and haemin yields from liver catalases

With the earlier method we found the ratio of bile pigment Fe to total haem Fe to be close to 0·25 for a number of preparations of ox and horse liver catalase of varying purity (Table 2). This suggested that catalase contained three protohaem groups and one bile pigment haem group in the molecule. With the more exact later method, however, a higher ratio was established, varying between 0·30 and 0·50 with an average of 0·38 (Table 3). This value does not suggest a stoichiometric ratio of bile pigment haem and protohaem, and the assumption of compounds between catalase and bile pigment haemoprotein, which contain one, two or three bile pigment haem groups in addition to three, two or one protohaem groups respectively, is thus not supported by experimental evidence.

Tables 2 and 3 show that the protohaem contents of the catalase preparations increase with their specific activities. Laskowski & Sumner [1941] have isolated crystalline ox erythrocyte catalase of the specific activity 48,000, and have found it to be free from bile pigment haem; the latter is, therefore, not necessary for catalase action. At first sight the experiments summarized in Tables 2 and 3 seem to support the view that the bile pigment haemoprotein is catalytically as active as the protohaem compound. The ratio of bile pigment Fe to total haem Fe does not vary with the specific activity over a wide range. A closer analysis reveals, however, that the bile pigment haemoprotein is inactive. The variations of the specific activity in our experiments are partly associated with factors other than that of the varying bile pigment content. The last column of Table 3 shows that the percentage of total haem Fe in catalase only occasionally approaches (e.g. preparations D 2*a*, D 2*c* and F 2 of ox liver catalase and D 1 of horse liver catalase) the theoretical value of 0·099 %, calculated for the presence of four haem groups in a catalase molecule of the molecular weight 225,000 [cf. Sumner *et al.* 1940]. The preparations with a lower total haem Fe content presumably still contain some inactive protein without haem groups, although some are crystalline. By multiplication with a correction factor (0·099 divided by percentage total haem Fe found) the true specific activity of the haemoproteins is obtained. If this is plotted against the ratio protohaem Fe/total haem Fe (Fig. 1), it is clearly seen that specific activity increases with decreasing bile pigment content. All but one of the points lie close to a straight line. This line cuts the 0-point, showing that the bile pigment haemoprotein is inactive, and reaches 52,000 for pure catalase without bile pigment haem. There is no significant difference between the activities of ox and horse liver catalases, and their variations with bile pigment haem content. The value 52,000 is close to that found by Sumner for the specific activity of ox erythrocyte catalase (48,000), which is free from bile pigment haem, and for the purest horse liver catalases.

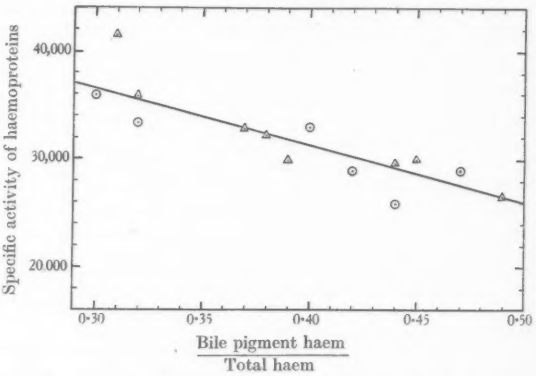

Fig. 1. Increase of specific activity with decreasing bile pigment haem content. △ Ox liver catalase. ⊙ Horse liver catalase.

Sumner *et al.* [1940] worked with a number of catalase preparations with more widely varying bile pigment haem contents, most of them of approximately equal purity, as the total haem Fe values (Table 2 of their paper) show. The specific activities, therefore, decrease with increasing bile pigment content without any correction for the presence of non-haematin compounds. It is more difficult to

Table 2. *Bile pigment and haemin yields from ox and horse liver catalases*

No.	Preparation		Specific activity	Protein conc. %	Haemin % of total protein	μg. per ml. Extracted proto-haem Fe			Bile pigment Fe	Ratio: bile pigment Fe / total haem Fe	*k*/proto-haem Fe
						Proto-haem Fe	proto-haem Fe	Bili-verdin			
			Ox liver catalase								
K2	Zeile	0·1%NH_3 eluate from Al_2O_3*	1,800	3·40	0·07	1·94	0·94	3·26	0·31	0·25	1,600
L1	,,	Crude extract	6,700	0·60	0·25	1·27	0·48	1·70	0·16	0·25	1,580
A2*b*	Sumner & Dounce	Eluted with NH_3 after A2*a*	10,700	0·90	0·22	1·70	0·84	3·10	0·30	0·26	2,640
C1	,,	Crystalline	—	—	—	—	9·32	33·5	3·21	0·25	—
A2	,,	,,	12,500	0·60	0·31	1·58	0·56	3·19	0·30	0·35	2,380
A2*a*	,,	A2 adsorbed to Al_2O_3 and re-eluted	—	—	—	1·57	0·81	3·81	0·36	0·31	3,120
A1	,,	Crystalline	17,500	1·90	0·31	5·06	3·25	13·0	1·25	0·27	3,260
B2	,,	Mother liquor of B1	17,000	0·90	0·33	2·57	1·67	4·7	0·45	0·21	2,960
B1	,,	Crystalline	—	—	—	—	8·45	36·4	3·48	0·29	—
B1′	,,	B1 recrystallized	33,000	1·20	0·73	7·53	4·28	14·4	1·38	0·24	2,630
			Horse liver catalase								
A1	Zeile	Crude extract	5,000	4·6	0·26	10·1	1·68†	4·6	0·44	0·21	1,140
A2	,,	After adsorption to Al_2O_3	25,700	3·82	0·50	19·2	12·3	46·4	4·43	0·27	2,560
B1	Keilin	Amorphous	20,000	4·28	0·48	20·7	9·9	44·0	4·20	0·28	2,080
B2*a*	,,	1st fraction of ammonium sulphate ppt.	—	—	—	20·8	11·6	33·6	3·21	0·22	—
B2*b*	,,	2nd fraction	—	—	—	17·0	9·0	32·4	3·10	0·25	—
C2	Zeile, Agner, Sumner	Crystalline	—	—	—	8·75	4·93	16·0	1·53	0·24	—

* Eluate after main part of catalase had been eluted with secondary phosphate.
† The small percentage of haemin recovery after splitting makes the result uncertain.

Table 3. *Bile pigment and haemin yields from ox and horse liver catalases*

No.	Preparation		Specific activity	Protein conc. %	Haemin % of total protein	μg. per ml. Extracted proto-haem Fe	Extracted proto-haem Fe	Bili-verdin	Bili-violin	Bile pigment Fe	Ratio: bile pigment Fe / total haem Fe	*k*/proto-haem Fe	Total haem Fe % of total protein
						Ox liver catalase							
H2	Sumner & Dounce	Inactivated extract*	2,500	2·07	0·66	11·75	4·70	14·7	20·9	3·40	0·42	220	0·098
H1	,,	Crystalline	—	—	—	1·18	0·33†	2·30	0·88	0·30	0·45	2,920	0·050
E2	,,	2nd fraction of crystals	15,000	1·60	0·36	4·96	2·16	12·3	1·5	1·32	0·38	2,510	0·050
E3	,,	Amorphous	19,700	0·62	0·43	2·28	0·81†	5·40	1·33	0·64	0·44	2,680	0·066
D1	,,	Amorphous	21,800	0·78	0·50	3·29	2·00	11·2	0·95	1·16	0·37	2,580	0·067
J1	,,	Crystalline	24,600	0·50	—	—	1·36	4·06	0·91	0·48	0·26	—	—
D2*c*	,,	,,	24,600	0·88	0·54	4·08	2·36	7·03	4·37	1·09	0·32	2,650	0·068
D2*b*	,,	,,	25,700	1·44	0·55	6·76	1·98†	15·0	6·9	1·94	0·49‡	2,740	0·093
E1	,,	,,	26,200	0·82	0·59	4·16	2·04	8·6	3·3	1·14	0·36	2,580	0·079
D2*a*	,,	,,	27,300	0·20	0·58	0·99	0·61	4·15	1·03	0·50	0·45	2,770	0·090
F2	,,	,,	36,400	0·90	0·70	5·39	3·45	11·9	4·3	1·55	0·31	3,040	0·087
						Horse liver catalase							
M1	Zeile, Agner, Sumner	Crystals	—	—	—	—	1·45	6·9	1·0	0·75	0·34	—	—
C2	,,	Mother liquor of crystals	—	—	—	—	3·42	28·8	6·2	3·35	0·49	—	—
G	,,	Crystalline	13,800	0·92	0·31	2·45	1·58	10·3	1·9	1·15	0·42	2,500	0·047
H	,,	,,	19,000	1·29	0·47	5·20	2·80	17·0	6·5	2·24	0·44	2,350	0·073
FI*b*	,,	,,	18,400	0·84	—	—	2·49	13·6	2·4	1·53	0·38	—	—
F2	,,	Amorphous	23,200	1·01	0·51	4·41	2·20	8·8	1·65	1·00	0·31	2,580	0·064
FI	,,	,,	24,000	1·95	0·51	8·53	4·22	24·0	4·2	2·69	0·39	2,750	0·072
FI*a*	,,	Crystalline	24,000	1·80	0·57	8·68	5·42	18·9	7·8	2·56	0·32	2,350	0·071
D1	,,	,,	28,000	1·32	0·60	6·75	1·84†	16·6	0·4	1·62	0·47	2,730	0·091
FII*a*	,,	,,	30,600	0·72	—	—	2·72	11·8	0·7	1·19	0·30	—	—
FII*b*	,,	,,	31,200	0·43	—	—	1·57	5·09	2·71	0·74	0·32	—	—

* After long standing of the crude extract; remarkably much inactive haematin and biliviolin.
† The small percentage of haemin recovery after splitting makes the result less certain.
‡ According to the absorption spectrum of the CO-haemochromogen: 0·38.

Table 6. *Increase of bile pigment haem in catalase by treatment with H_2O_2 in the presence of ascorbic acid*

Liver catalase: Species	Vol. ml.	*k*	Ascorbic acid mg. per 100 ml.	*t*°	H_2O_2 g.	mg. H_2O_2 / mg. active enzyme	Bile pigment Fe / Total haem Fe: Before oxidation	After oxidation	Total activity decrease %	Activity decrease due to oxidation of prosthetic group %	*dC*/*dE* mg. H_2O_2 destroyed / mg. enzyme inactivated	Specific activity: Before oxidation	After oxidation	Decrease %	*k*/protohaem Fe: Before oxidation	After oxidation
Ox	15	6,150	5	30	62·5	1,700	0·26	0·58	83	44	2,000	24,600	18,300	25	—	—
Horse	10	12,200	5	30	45	900	0·44	0·49	65	9	1,400	—	—	—	2,350	1,650
Horse	10	4,530	5	30	21	1,200	0·32	0·52	49	30	2,350	27,000	23,100	15	2,590	1,850
Horse	22	6,340	25	5	66	1,200	0·42	0·48	34	10	3,300	13,800	13,000	6	—	—
Horse	22·2	6,340	30	23	67·5	1,200	0·42	0·46	19	7	6,500	—	—	—	2,500	2,400
Horse	20	6,340	40	37	30	600	0·42	0·43	74	2	800	—	—	—	2,500	2,090

draw a straight line through the points of Sumner's table in a diagram similar to Fig. 1, with or without correction for presence of non-haematin compounds, and the line does not appear to go through the 0-point. This is probably owing to the way in which the bile pigment haem Fe is determined by Sumner as 'blue iron' fraction. Even the best catalase preparations still contain small amounts of ferritin, a protein with high iron content, and it is likely that a small part of the ferritin iron is not precipitated with the protein, but is determined as 'blue iron'. Our method takes into consideration only haem and bile pigment haem, and the presence of iron in any other form, e.g. as ferritin, does not interfere with the results. Apart from this minor difference, however, our results independently confirm those of Sumner by different methods.

Spectrophotometric study of the catalase haemochromogen

While Zeile [1933] claimed that the haemochromogen prepared from catalase is identical with that from protohaem, Lemberg & Wyndham [1936–7] observed an absorption band at 651 mμ with the pyridine haemochromogen prepared from highly active, but amorphous horse liver catalase.

The same band is observed, when crystalline preparations of ox and horse liver catalase are studied. NaOH, final conc. 1%, is added to catalase and after the addition of a few mg. $Na_2S_2O_4$ the absorption spectrum is immediately observed. With the spectrophotometer the ratio $\epsilon_{650} : \epsilon_{557}$ is found to be 0·25, while for protohaemochromogen, prepared in the same manner from haemoglobin, this ratio is only 0·015. This absorption in the red part of the spectrum is not due to unspecific background absorption, as Stern [1937] assumed, since a definite maximum of absorption at 650 mμ is found. It cannot be due to biliverdin either, since biliverdin is reduced by $Na_2S_2O_4$ in alkaline solution to a yellow compound which does not absorb in the red.

The presence of another haemochromogen in addition to protohaemochromogen is confirmed by examination of the CO-haemochromogen. By bubbling coal gas through the solution, previously saturated with the gas, during the addition of $Na_2S_2O_4$ and NaOH, the danger of secondary oxidation by H_2O_2 formed from $Na_2S_2O_4$ is prevented. A strong absorption band at 630 mμ is found, which is usually about half as high as that of CO-protohaemochromogen at 570 mμ (Fig. 2).

The absorption curve differs considerably from that of CO-protohaemochromogen prepared from haemoglobin.

While the haemochromogen accompanying protohaemochromogen is similar to cholehaemochromogen in some properties, differences exist between them. The position of the absorption band of the CO-compounds is the same, and removal of CO causes the same shift to 618 mμ; the absorption band at 630 mμ of the catalase compound is, however, accompanied by absorption rising towards the infra-red. A further difference is that choleglobin gives only a poor yield of biliverdin, while bile pigment formation from the catalase haemoprotein appears to be quantitative. While such differences

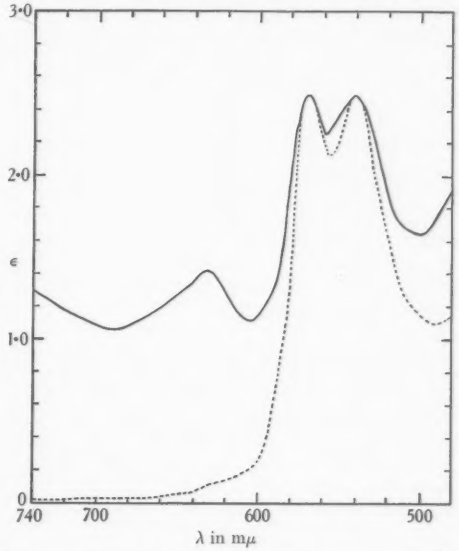

Fig. 2. CO-haemochromogen absorption curve. —— CO-haemochromogen from catalase. ---- CO-haemochromogen from haemoglobin.

may be due to the different proteins to which the bile pigment haem group is linked, the fact that the absorption band of the haemochromogen before the action of CO is found at 650 mμ, not at 618 mμ, suggests a prosthetic group in catalase slightly different from cholehaem. It seems to constitute a link between verdohaem and cholehaem.

The bile pigment haemochromogens have been found to possess approximately equal molar extinction coefficients at the respective maxima of absorption. We may therefore assume that the CO-haemochromogen from catalase has the same molar extinction at 630 mμ as CO-cholehaemochromogen, and can then calculate the ratio of bile pigment haem to total haem as

$$(\epsilon_{630}/\epsilon_{570} - 0{\cdot}04)/(0{\cdot}55\epsilon_{630}/\epsilon_{570} + 0{\cdot}96).$$

This formula is in principle the same as that given by Lemberg *et al.* [1941*a*], except that it gives the ratio bile pigment haem/total haem instead of bile pigment haem/protohaem as in the earlier formula.

Table 4 shows that the ratio found in this way is the same as that found by determinations of bile pigment and haemin in the split products. With one exception (D 2*b*) the results agree very well. It can

Table 4

Molar ratio : $\frac{\text{bile pigment haem}}{\text{total haem}}$

Ox liver catalase preparation	Found by estimation of the split products	Found by spectrophotometric analysis
F2	0·31	0·35
D2*c*	0·32	0·33
E1	0·36	0·38
D1	0·37	0·40
E2	0·38	0·38
L	0·40	0·42
E3	0·44	0·44
H1	0·45	0·43
D2*a*	0·45	0·43
D2*b*	0·49	0·38

be concluded from these experiments that the biliverdin obtained from catalase by splitting with acids, occurs in catalase in the form of a bile pigment haemoprotein.

OXIDATION OF CATALASE TO BILE PIGMENT HAEMOPROTEIN BY H_2O_2

Calculation of the minimum amount of H_2O_2 required. We have reported [Lemberg *et al.* 1939] that we could not observe an increase in the bile pigment haem content of catalase when we allowed the enzyme to destroy gradually a large amount of H_2O_2.

From the kinetics of the enzyme reaction given above we can now calculate how much H_2O_2 catalase can destroy before it is inactivated, and thus determine the minimum amount of H_2O_2 with which the enzyme must react in order to obtain an experimentally verifiable increase in its bile pigment haem content. These calculations show that the amounts of H_2O_2 required are so large that the experimental difficulties are insurmountable, even if the inactivation of catalase in dilute solution were due entirely to the oxidation of protohaem to bile pigment haem.

From the two equations

$$-dC = kEC\,dt \quad \text{and} \quad -dE = k'EC\,dt$$

it follows that

$$\frac{dC}{dE} = \frac{k}{k'} = \frac{A + C_0}{E_0},$$

where $A + C_0$ is measured in terms of ml. $KMnO_4$/10 ml. H_2O_2 solution. C_0 ml. $KMnO_4$ are equivalent to 10 ml. $N/100$ H_2O_2 and $A + C_0$ therefore to $(A + C_0)/100C_0$ mol./l. E_0 in mol./l. is determined as $20k_0/225{,}000K$, where k_0 is the initial velocity constant of H_2O_2 destruction, and K the specific activity. Since we are interested only in the oxidation of the active catalase molecules, the maximum specific activity (50,000) is used. Thus we have:

$$\frac{dC}{dE} = 5{\cdot}62 \cdot 10^6 \frac{A + C_0}{C_0 k_0} \frac{\text{g. mol. } H_2O_2 \text{ destroyed}}{\text{g. mol. catalase inactivated}}$$

$$= 850 \frac{A + C_0}{C_0 k_0} \frac{\text{g. } H_2O_2 \text{ destroyed}}{\text{g. enzyme inactivated}}$$

or $$850 \frac{1}{C_0 k'} \frac{\text{g. } H_2O_2 \text{ destroyed}}{\text{g. enzyme inactivated}}.$$

k' was not determined with great accuracy; at 0° it varied from 0·0007 to 0·0023 with an average of 0·0014. No significant difference was found between the k' of ox and of horse liver catalase. dC/dE varies from 20,000 to 75,000 g. H_2O_2/g. catalase, with an average of 35,000.

In order to inactivate 10 mg. out of 50 mg. active enzyme (the minimum needed for estimation) more than 1100 ml. of 30 % H_2O_2 solution would have to be added. This would increase the ratio of bile pigment Fe to total haem Fe from 0·40 to 0·52, provided that the inactivation of the enzyme were entirely due to the oxidation of haematin to bile pigment haematin.

Effect of ascorbic acid on the destruction of catalase by H_2O_2. We have found, however, a way to overcome these difficulties. Various authors have observed that the action of catalase is inhibited by reducing substances, e.g. glutathione and ascorbic acid. Marks [1936] found that ascorbic acid and reduced glutathione increase the rate of inactivation of catalase by atmospheric O_2. By the action of ascorbic acid and H_2O_2 haemoglobin and haemochromogens are oxidized to bile pigment haem compounds. We have therefore investigated whether the inhibition of the enzyme action by ascorbic acid is due to a real diminution of k, or to a faster destruction of the enzyme by H_2O_2 (increase of k').

Ascorbic acid reacts only slowly with dilute H_2O_2 or $KMnO_4$. If the first drop of $N/100$ $KMnO_4$ solution giving a colour lasting for more than 10 sec. is taken as the end-point, the presence of ascorbic acid in a concentration not greater than 10 mg./100 ml. does not interfere with the titration. Moreover there is no measurable reaction between H_2O_2 and ascorbic acid during 10 min.

Table 5 shows that k_0 is decreased little, if at all, by ascorbic acid, while k' is greatly increased. Ascorbic acid inhibits the enzyme action by accelerating the destruction of catalase by H_2O_2. The ratio dC/dE is thus diminished from 35000 to 4000 by a concentration of 10 mg. ascorbic acid/100 ml.

Experiments with glutathione failed to yield clear-cut results. Reduced glutathione reacts too rapidly with H_2O_2, and also with $KMnO_4$. Nevertheless our experiments indicate that the inhibition of catalase activity by reduced glutathione is not due to increased destruction of the enzyme.

Table 5. *Destruction of catalase by H_2O_2 at 0° in the presence and absence of ascorbic acid*

	k_0	k'	$\frac{dC/dE}{\text{g. } H_2O_2 \text{ destroyed}}{\text{g. catalase inactivated}}$
Horse liver catalase without ascorbic acid	0·054	0·0018	29,000
Horse liver catalase with 2 mg./100 g. ascorbic acid	0·054	0·0074	7,100
Horse liver catalase with 10 mg./100 g. ascorbic acid	0·054	0·0127	4,100
Ox liver catalase without ascorbic acid	0·056	0·0017	27,000
Ox liver catalase with 4 mg./100 g. ascorbic acid	0·052	0·0088	4,700

Large-scale experiments. These were made to see if treatment of catalase with H_2O_2 in the presence of ascorbic acid increases the bile pigment haem content. Much higher concentrations of catalase and, at least locally, of H_2O_2 cannot be avoided in these experiments. Otherwise the large dilution of the enzyme would make impossible its recovery in the concentrated form required for bile pigment estimations. Even the use of concentrated (30 %) H_2O_2 causes much dilution. Concentrated H_2O_2, however, destroys the enzyme by denaturing the protein. The danger of this was minimized by gradual addition and thorough mixing.

30 % H_2O_2 (Schering Kahlbaum *pro analysi*) was neutralized with NaOH and slowly dropped from a micro-burette, at a rate of one drop every 7–10 sec., into the dilute well-buffered (*p*H 6·8) catalase solution containing the ascorbic acid. The mixture was kept in a continuous swirling motion, and the drops of H_2O_2 were not allowed to fall into the froth; gentle swirling by hand was best. Occasionally octyl alcohol had to be added, but this caused some enzyme denaturation and was avoided if possible. It can be calculated that with the enzyme concentrations used all the H_2O_2 was almost completely destroyed before the next drop was added.

In the preliminary experiments, to 0·5 ml. of a catalase solution ($k = 7000$) 5 ml. of 30 % H_2O_2 were added as described; temp. $< 5°$. In a second experiment ascorbic acid was added (5 mg./100 ml.). Without ascorbic acid 26 % of the enzymic activity was destroyed ($dC/dE = 4200$), with ascorbic acid 59 % ($dC/dE = 1800$). Ascorbic acid thus increased the destruction of catalase by H_2O_2 under these conditions, but not as much as in the experiments in very dilute solutions (cf. Table 5). This suggests that in the large-scale experiments much catalase is destroyed in a different way, a way which is much less or not at all accelerated by ascorbic acid. If it is assumed that the enzyme destruction in dilute solution in the presence of ascorbic acid is entirely due to the oxidation of protohaem to bile pigment haem and this reaction itself remained unaltered in the large-scale experiments, it can be calculated that the destruction of 60 % of the activity of the enzyme in the experiment above ought to be accompanied by a significant increase in the bile pigment haem content. dC/dE in the large-scale experiment (1800) is about one-third of dC/dE in dilute solution (4700, cf. Table 5), i.e. three times as much enzyme is destroyed by the same amount of H_2O_2 in the large-scale experiments as in dilute solution, in which we assume the destruction to be due to the oxidation of protohaem to bile pigment haem. If, of the 60 % total loss of activity, 20 % is due to oxidation of protohaem to bile pigment haem, an original ratio of bile pigment haem to total haem of 0·40 ought to be increased to 0·52, which is far beyond the range of experimental error. The large-scale experiments were carried out at temperatures from 5 to 37°. Since the inactivation of the enzyme by H_2O_2 has a higher temperature coefficient than the catalytic destruction of H_2O_2, higher temperatures ought to be favourable, up to the critical zone, where temperature inactivation of the enzyme becomes significant. After the addition of H_2O_2 the mixtures were worked up in the following way. The catalase activity was determined as usual. For the other estimations the catalase was concentrated by precipitation with $(NH_4)_2SO_4$. In spite of being finely dispersed the precipitate was easily collected on a Whatman no. 42 filter; the water-clear filtrate was practically inactive. The catalase was dissolved in secondary phosphate solution and dialysed at 0° in a collodion bag.

The experiments summarized in Table 6 (p. 122) show that in five of six experiments a definitely higher bile pigment haem content was found after treatment with H_2O_2. In the one experiment in which no significant increase was found, H_2O_2 had been added more rapidly at 37°, and the fact that a smaller amount of H_2O_2 had led to the same degree of enzyme destruction (see columns 5 and 8), indicates greater denaturation of protein.

The quotient dC/dE and that part of enzyme inactivation due to the oxidation of protohaem to bile pigment haem vary considerably. This is not surprising in view of the experimental difficulties. The degree of denaturation of catalase by conc. H_2O_2 may alter according to froth formation, addition of octyl alcohol and efficiency of mixing. The ascorbic acid concentration is altered during the addition of H_2O_2 by dilution and by oxidation, and while the former was avoided by adding ascorbic acid from time to time, the degree of the latter was unknown.

We had expected that these experiments would supply further evidence that the bile pigment

haemoprotein is catalytically inactive, but no conclusive evidence was obtained. The determination of protohaem as haemochromogen is more difficult than with untreated catalase and is disturbed by cloudiness of the solution and incomplete reduction by $Na_2S_2O_4$. If the values for protohaem are correct, H_2O_2 causes a fall of the quotient k/protohaem Fe, i.e. some inactivation of catalase without precipitation or oxidation, and the picture is further complicated by the fact that the decrease of the specific activities of the recovered solutions is smaller than would correspond to the decrease of total activity. The latter must be explained by the denaturation and removal of inactive proteins in subsequent treatment.

No difficulties were, however, encountered in the determination of protohaem after acid splitting and of the ratio bile pigment Fe to total haem Fe. Duplicate estimations of this ratio gave results within $\pm 0{\cdot}02$ and usually $\pm 0{\cdot}01$. With the exception of the experiment in the last row of Table 6, which may be considered as a blank, the increases of the bile pigment haem content are significant.

DISCUSSION

In earlier experiments we have found that by the action of H_2O_2 in the presence of ascorbic acid the protohaem group of haemoglobin and of haemochromogens is oxidized to bile pigment haem. Analogous reactions with a slightly modified mechanism (coupled oxidation), but of principally the same type, occur *in vivo*. They underlie the physiological formation of bile pigment from haemoglobin as well as the intracorpuscular biliverdin formation by phenylhydrazine [Lemberg & Legge, 1942] and other drugs. Ox and horse liver catalases contain bile pigment haem, while ox erythrocyte catalase has been found by Laskowski & Sumner [1941] to contain protohaem alone. Does catalase undergo an oxidation *in vivo* similar to haemoglobin?

In the present paper we have shown that by the action of H_2O_2 in the presence of ascorbic acid the protohaem groups of catalase are oxidized to bile pigment haem groups. These experiments had to be carried out with unphysiologically high concentrations of enzyme and H_2O_2 but we have shown the effect of ascorbic acid on the rate of the enzyme destruction in low concentrations, and it is likely that the mechanism is the same.

The bile pigment haem content of the liver catalase can be explained in three ways: (1) it is an artefact and due to the oxidation of catalase in post-mortem changes or during its isolation from the liver; (2) bile pigment haem groups formed by the breakdown of haemoglobin in the liver are incorporated in the synthesis of catalase; or (3) catalase itself undergoes an oxidation in the liver.

With regard to the first possibility, it might be argued that the presence of larger amounts of ascorbic acid in the liver than in the red cells may explain why an oxidation does not occur during the isolation of catalase from erythrocytes. By autoxidation of ascorbic acid H_2O_2 is formed and may act together with ascorbic acid on liver catalase which was originally free from bile pigment haem. The amounts of H_2O_2 which can be formed during the isolation cannot, however, be large and appear to be insufficient to explain an oxidation of 30–50% of the prosthetic group. Even at 20° the inactivation of catalase by ascorbic acid is slow [Marks, 1936] and the isolation is carried out at 0°. Neither Sumner *et al.* [1940] nor we ourselves have found evidence supporting this assumption.

The second explanation does not appear probable if it is remembered how specific is the synthesis of haemoglobin. Porphyrins with side chains differing from those of protoporphyrin are not united with iron and globin, even though such compounds may still carry O_2, while if the catalase haemoprotein were synthesized from bile pigment haem and catalase protein, it would be inactive.

This leaves the third hypothesis, an *in vivo* oxidation of catalase, as the most likely one. The difference between erythrocyte and liver catalases would be explained by the greater content of reducing substances in the liver cells and their greater respiration.

If this is the correct explanation, it leads to important implications. Catalase can then no longer be considered as a mere safety valve against occasional H_2O_2 formation. H_2O_2 must be destroyed by the enzyme *in vivo* on a scale which is not negligible. Even in the presence of ascorbic acid in concentrations found in the liver cells, each molecule of catalase must have destroyed at least 2×10^7 molecules of H_2O_2 before it became converted into bile pigment haemoprotein. 1000 g. of horse liver contain 0·5–1·0 g. of active protohaem catalase according to the activity of the extracts [Stern, 1935–6], which would mean the destruction of at least 500–1000 g. of H_2O_2 by 1000 g. of liver during the lifetime of the enzyme. We have not yet any definite data on the latter, but to judge from the fact that the liver catalase of a colt did not contain less bile pigment haem than that of the adult horse [Sumner *et al.* 1940] it is probably to be measured in weeks or months rather than in years.

The method of determining the bile pigment content of catalase may be used for studying the metabolism of catalase, but since large amounts of catalase are required for the estimations, other methods, e.g. with tracer elements, would be preferable.

Finally our experiments lend support to the findings of Keilin & Hartree [1938] that ferric

catalase iron is reduced to ferrous iron during its action on H_2O_2. Only ferrous haem compounds are oxidized by H_2O_2 to bile pigment haem compounds. Ascorbic acid does not reduce catalase, but may protect the ferrous H_2O_2-catalase compound against autoxidation.

SUMMARY

1. A simplified procedure for the isolation of crystalline horse liver catalase is described.

2. By splitting with acetic acid and extracting with ether, biliverdin and small amounts of biliviolin are obtained from crystalline ox and horse liver catalases.

3. The molar ratio of (bile pigment)/(bile pigment + haem) from impure and pure horse and ox liver catalases was found to vary from 0·3 to 0·5.

4. The CO-haemochromogens of these catalase preparations had, in addition to the absorption bands in CO-protohaemochromogen, an absorption band in the same position as that of CO-cholehaemochromogen. Spectrophotometric analysis showed that the height of this band was proportional to the bile pigment content as found by the splitting with acids, and that the same values for the ratio of (bile pigment Fe)/(bile pigment Fe + haem Fe) were found by both methods. This shows that the bile pigments are derived from a bile pigment haemoprotein.

5. If the presence of inactive haem-free protein in the impure preparations is taken into consideration, it can be shown that the specific activity (Kat.-f.) increases with decreasing bile pigment haem content, being about 50,000 for catalase containing protohaem only, and nil for the bile pigment haemoprotein.

6. A theoretically sound method for measuring catalase activity is described, which gives also the rate of destruction of the enzyme by H_2O_2.

7. Ascorbic acid inhibits catalase by accelerating the destruction of the enzyme by H_2O_2.

8. By the action of H_2O_2 on catalase in the presence of ascorbic acid, bile pigment haemoprotein is formed from protohaem catalase.

9. The significance of these findings for the explanation of the bile pigment haem content in liver catalase is discussed.

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The Technique of the Biological Vitamin A Assay

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The publications of Burn [1937], Irwin [1937], Coward [1938] and Bliss & Marks [1939], to cite a few of the more important papers, testify to the existence of a good measure of agreement on the general principles of the graded-response biological assay. At the same time assays of any particular substance usually necessitate the use of some *ad hoc* technique. The rat-growth (curative) vitamin A

assay is a notable example, and we here outline recent developments in the conduct of this assay in our laboratory.

Some special considerations regarding the vitamin A assay are listed below:

(i) The criterion of vitamin A depletion in the experimental animals is cessation, or marked retardation, of growth, a condition liable to cause secondary reactions that may jeopardize the response to vitamin A.

(ii) The useful range of the growth-response curve is limited at its lower end by an abrupt fall at the point where the dose is just insufficient to prevent the onset of a pathological state, with consequent invalidity of response, i.e. the curve does not rise smoothly from zero.

(iii) If the behaviour of the rats during the depletion period is 'good' there will be occasional cases of death or loss of weight. There is no biometrically acceptable method of incorporating the records of such animals into the assay: if the rats die during the test period there is clearly no alternative to the somewhat unsatisfactory procedure of, in effect, ignoring the existence of the behaviour records; and because of this, the records of rats that lose weight have also, *a fortiori*, to be set aside. Otherwise we should have the paradox that while a negative growth pulled down the group average, loss of weight culminating in early death left the average undisturbed. It is therefore desirable that the assay conditions be such that there is a maximum number of positive growth responses at all dosage levels.

(iv) The International Standard is not vitamin A but the provitamin β-carotene. Some assurance that the two substances give a common kind of response under all conditions of the assay is therefore desirable, i.e. evidence of the constancy of the conversion of β-carotene to vitamin A is required. There is therefore need to demonstrate a substantial identity between the normal variations of response, and between the constants of slope of the curves of response, to Standard and test materials.

The inaugural work on our assay technique has been described [Morgan, 1934]. Minor changes have since been introduced, e.g. the vitamin A-deficient diet now in use is:

	%
Casein A/E	10
Coconut-cake meal	30
Flaked rice	48
Dried yeast	8
Steenbock's no. 40 salt mixture	4
	100

The addition of vitamin D has been shown to be unnecessary with this diet. The animals (bucks only) become depleted of vitamin A at the age of about 50 days, litters always *en bloc*, so that the members of each litter are started on the 3-week test on the same day. Typical average behaviour on the diet is portrayed in simplified form in Fig. 1.

Morgan [1934] found that for the then existing regime the logarithm of the dosage was linearly related to the square of the growth response. In practice the differences between the squares of

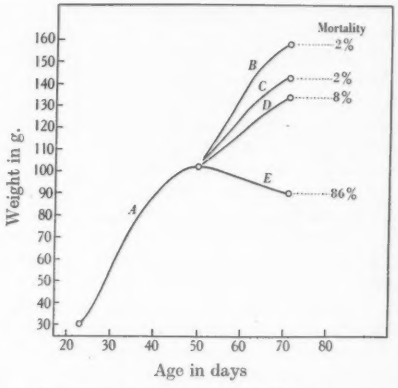

Fig. 1. Composite growth curves of 834 recent test rats. Curve A. From weaning to vitamin-A-depletion point. Curve B. Test period; positive controls; on +20 i.u./day. Curve C. Test period; on 2 i.u./day. Curve D. Test period; on 1·2 i.u./day. Curve E. Test period; negative controls; no vitamin A.

response in weight-matched, isogenic, Standard (T): test substance (S) pairs were taken as metameters* with which to calculate directly $\sigma_{(S-T)}$ and to evaluate the 'dose ratio' (S/T), and hence the test-material potency, by substitution in

$$G_S^2 - G_T^2 = b \log S/T.$$

Until recently we have continued to use this equation. At approximately 6-monthly intervals the accumulated test data, covering a dosage range of about 0·5–3·0 i.u./day, were summarized to check the validity of the equation and to recalculate b. There is, of course, no *a priori* reason why the growth: log-dose curve should be linear in the vitamin A, or in any other, biological assay; the important consideration is whether accuracy is enhanced by the employment of a precise equation rather than the convenient linear approximation.

One of the consequences of the above technique was that the necessity of pairing left an unusable residuum of 10–15% of the animals. This wastefulness caused us to investigate the rationale of rat selection; at the same time the biometry of the assay was reviewed, special attention being given to the stability of equation constants.

* Metameter = the measurement used in a biological assay (see *Biochem. J.* 1942, **36**, 411, footnote).

To investigate the pairing technique, the records of six routine assays were analysed. In anticipation of this work the usual strictness of weight matching ($\gtrless \pm 4$ g.) of the isogenic pairs had been relaxed. From each set of records two correlation coefficients were calculated, first, that within the paired Standard : test-material growth-responses, and secondly, that between initial-weight differences and growth-response differences in the individual pairs —after segregation of interlitter and interdose differences (see Table 1).

Table 1. *Correlations (a) within isogenically paired comparisons of growth-response and (b) between initial-weight differences and subsequent growth-response differences in the same pairs*

Assay no.	No. of (buck) pairs	(a) Correlation coefficient r_a	(b) Correlation coefficient r_b
i	27	+0·497	+0·024
ii	24	+0·302	+0·201
iii	21	+0·387	+0·118
iv	23	+0·474	−0·404
v	32	+0·478	+0·195
vi	20	+0·293	−0·010
Means (by z transformation)		+0·418	+0·030

The mean r_a is highly significant and indicates that the pairing technique is advantageous, while the mean r_b is not significant and indicates that weight-matching is superfluous [cf. Bacharach, 1940]. Thus isogenic pairing, or, in general, intralitter comparison, is the important factor. A corollary (obtained from the weighed means of the variance ratios) is that 10 pairs of isogenically matched rats give results comparable in accuracy with those of 19·3 arbitrarily selected pairs.

We looked, therefore, for a new assay plan. We observed that if a group of test animals is equally divided between four dosages, two levels of Standard and two of test material, the use of the 'internally' calculated best linear equation (growth : log-dose) automatically compensates for real deviations from the assumed relationship. To illustrate: suppose that the true relation between dosage (D) and growth response (G) obtaining in a group of rats used in an assay were expressible as the polynomial

$$G = 27 + 88 \log D - 60 (\log D)^2$$

(the formal expression of a typical 'square of the growth' equation such as we encounter), and that in the assay there were equal numbers of animals on 1 unit and 2 units Standard (S) and nominal 1 unit and 2 units test-material (T); ideally we can imagine the exclusive use of isogenic tetrads of bucks for this four-term dosage distribution. Suppose further that the true dose ratio T/S was 1·4 so that with a sufficiently large number of animals the growth responses would approximate to the following (calculated from the polynomial):

1 unit S : 27·00 g.
2 units S : 48·05 g.
Nominal 1 unit $T \equiv 1{\cdot}4$ units S : 38·58 g.
Nominal 2 units $T \equiv 2{\cdot}8$ units S : 54·35 g.
All doses 'daily'.

Let us now ignore the true growth-dosage equation and interpret the assay from the best internal linear equation. We have

Slope b, from Standard growths $= 21{\cdot}05/\log 2 = 69{\cdot}9$
Slope b, from test-material growths $= 15{\cdot}77/\log 2 = 52{\cdot}4$

The two values are, of course, significantly different. If, however, they are averaged and the mean (61·2) is used to evaluate the (supposedly unknown) dose ratio from the linear equation, we have

$$\text{Dose ratio} = \text{antilog}\ \frac{\frac{1}{2}(38{\cdot}58 - 27{\cdot}00) + \frac{1}{2}(54{\cdot}35 - 48{\cdot}05)}{61{\cdot}2} = 1{\cdot}40.$$

This compensatory effect operates over fairly wide limits: it certainly covers all curves in our experience. An incidental inference is that a statistical demonstration of departure from parallelism in a biological assay is not necessarily evidence of the invalidity of the test.

When the Standard : test-material dose ratio is unity the equation constants are of minimum importance in assay interpretation, so that the smaller the dose ratio the better the assay. Consequently we always repeat assays in which the dose ratio is found to be unexpectedly high or low; in the repetition appropriately readjusted test-material doses are used.

Because isogenic tetrads comprise only a few of the available test animals, others must also be used for routine work. The following scheme utilizes all rats to advantage:

Dosage levels:
1·2 and 2·0 units of Standard (S)
'nominal' 1·2 and 2·0 units of test-material (T)

Distributions:
In a four-buck litter: $S_{1\cdot2}$, $S_{2\cdot0}$, $T_{1\cdot2}$ and $T_{2\cdot0}$
In a three-buck litter: $S_{1\cdot2}$, $S_{2\cdot0}$ and $T_{1\cdot2}$ or $S_{1\cdot2}$, $S_{2\cdot0}$ and $T_{2\cdot0}$ or $S_{1\cdot2}$, $T_{1\cdot2}$ and $T_{2\cdot0}$ or $S_{2\cdot0}$, $T_{1\cdot2}$ and $T_{2\cdot0}$ } equally divided between all such litters
In a five-buck or more litter: any suitable extension of the smaller distributions

Thus each test litter yields at least one $S : T$ comparison and one b value. Litters of two bucks are used for negative and positive controls only. Intralitter initial weight differences are evenly spread between doses as a precautionary measure against the emergence of a correlation between initial weight and growth response.

The two dosage levels, 1·2 and 2·0 i.u., are not, of course, integral to the scheme; they are purely

arbitrary. The choice of a dosage interval is influenced by the fact that the smaller it is the less frequently will responses fall outside the critical range (this is especially important in assays of materials that are unexpectedly high or low in potency), whereas the larger the interval is the more sensitive and, other things being equal, the more accurate the assays will be. Our values are a working compromise arrived at by accumulated experience coupled with certain dosimetric considerations.

The object of the scheme is to have available at the end of the test the following four, equally weighted, isogenic pair-difference means of growth response:

$(S_{1\cdot2} - T_{1\cdot2})$ and its S.E.$\}$ $(S_{2\cdot0} - T_{2\cdot0})$ and its S.E.$\}$ hence overall $(S-T) \pm \epsilon_{S-T}$

$(S_{2\cdot0} - S_{1\cdot2})$ and its S.E.$\}$ $(T_{2\cdot0} - T_{1\cdot2})$ and its S.E.$\}$ hence overall $b \pm \epsilon_b$

Concentration upon pair differences of response as the metameters *ipso facto* eliminates interlitter variations, and the given arrangement enables this to be done thoroughly despite the limitations and variations of litter size. From the data the assay and the approximate error (incorporating the error of the slope b) can readily be calculated. Furthermore, in a series of assays, fluctuations of b can be followed and any significant differences between the b's of Standard and test material detected.

More than 30 assays have been carried out to date on this plan. In several of the larger ones enough tetrad (four buck) litters were incorporated to permit analysis of variance of growth response and of covariance of initial weight and growth response. Nine such calculations have been made (Table 2).

Note, first, the renewed emphasis on interlitter variations; secondly, that there is further evidence of the negligible influence of initial weight on subsequent growth response; thirdly, that in only one case, that of assay 454, does departure from parallelism approach statistical significance, i.e. the ($P = 0{\cdot}95$) level of variance ratio for the particular circumstances is 4·2, whereas in this assay it is, at most, 4·8; fourthly, that in all cases but one, variations due to slope are highly significant, assay 465 being a borderline exception.

For the purposes of statistical analysis these part assays have each been evaluated from 'internal' estimates of b. In practice, however, this is unsatisfactory. The sampling error of a single-assay estimate of the slope is large. And though the error of an estimate from a number of assays is, of course, correspondingly small, the use of such an estimate would be justified only if it were proven that absolute changes in b did not take place within the covered period of time. In the case of these rats it was found that absolute changes did take place

Table 2. *Analysis of variance and covariance of growth response (GR) and initial weight in nine vitamin-A part-assays composed of isogenic tetrads of buck rats on four different doses, two of standard* (S) *and two of test material* (T)

Assay no.	No. of isogenic tetrads	Calculations based on GR taken (*a*) alone and (*b*) adjusted for init. wt.	Residual σ of GR	Variances of GR due to three specified factors expressed as multiples of the corresponding variances due to residual error: Diff. between litters	Slope b	Departure from parallelism	Coefficient of regression of GR on init. wt.	'Unadjusted' assay as % of 'adjusted' assay	Upper limit of error ($P = 0{\cdot}95$) expressed $\equiv 10$ pairs %
454	11	(*a*)	4·66	2·44	26·76	4·28	−0·563	96·3	140·1
		(*b*)	4·36	2·80	24·73	4·82			143·6
455	17	(*a*)	4·79	3·44	20·77	0·04	−0·125	101·7	150·0
		(*b*)	4·78	3·53	20·95	0·06			149·6
459	7	(*a*)	3·93	13·77	9·19	2·23	−0·123	99·8	160·6
		(*b*)	3·95	13·89	9·74	2·13			158·5
460	9	(*a*)	5·73	2·29	11·78	0·12	−0·201	105·4	151·2
		(*b*)	5·30	1·43	15·84	0·08			141·6
465	9	(*a*)	7·35	2·06	4·35	0·02	−0·115	100·9	206·7
		(*b*)	7·50	2·26	4·04	0·02			211·4
469	11	(*a*)	6·62	3·53	53·17	0·03	+0·297	102·3	133·5
		(*b*)	6·55	3·99	57·54	0			131·1
472	9	(*a*)	7·53	2·25	23·91	0·11	−0·253	105·8	140·9
		(*b*)	7·58	1·55	28·29	0·11			134·9
474	10	(*a*)	6·96	1·78	40·46	1·40	−0·126	100·2	125·8
		(*b*)	7·02	1·63	40·62	1·00			125·7
481	9	(*a*)	4·73	2·11	25·39	1·89	−0·900	97·7	133·0
		(*b*)	4·77	2·13	23·22	1·78			135·5
Weighted means (92 total)		(*a*)	5·89	3·44	24·89	1·05	−0·142	101·1	150·0
		(*b*)	5·85	3·44	25·85	1·05			149·4

and it was therefore thought advisable to use the weighted mean of the estimate of b for any given assay plus those of the few (latterly two) immediately preceding assays: a balance is thereby struck between avoidance of short-term apparent variations of b on the one hand and recognition of real variations over longer periods on the other. Since the introduction of the new regime, the moving averages employed have revealed definite variation trends. All these averages are charted in Fig. 2.

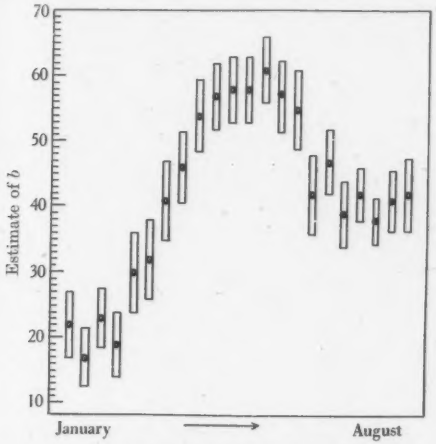

Fig. 2. Variation in b, the constant of slope in the rectilinear growth/log-dosage equation during the current year. Mean and limits of S.E. are given. The whole constitutes an assay to-assay moving average, each estimate being based on the data of the particular assay together with those of (generally) the two preceding assays—an average of 62 values. (By one 'value' of b is meant the estimate from one isogenic pair difference of response.) The mean interval between assays—and therefore between estimates of b—is 8 days.

It is clear from Fig. 2 that real variations of slope have occurred. If the component series of separate assay estimates is roughly trisected the following averages are obtained (Table 3):

Table 3. *Variations in* b, *the constant of slope*

	Mean b	Standard error
Earliest group (158 values)	24·5	3·23
Middle group (173 values)	57·5	3·43
Latest group (157 values)	42·0	2·95
488 values	41·8	

It is easily demonstrable that any one of these three means is significantly different from the other two. Thus there is danger in using a fixed b over even a few months.

Long-period fluctuations in b are of interest. The mean annual estimates over the past few years (recalculated from the records, because until recently the 'square-growth' b was in use) are given below, together with the responses to 2 units/day and the mean ($P=0·95$) error of a 10-pair assay in which the growth responses to nominally equivalent dosage levels of Standard and test material are equal and the calculated dose-ratio is therefore unity. Under these conditions the error is at a minimum.

Table 4. *Annual fluctuations in certain mean values in vitamin A assays*

Year	Mean growth on 2 units Standard g.	Mean b from isogenic pair-differences only (No. of values)	S.D. of pair-differences of growth $\sigma_{(S-T)}$	Upper limit of error ($P=0·95$) for a 10-pair assay with equal S and T growth responses %
1936	45	48 (141)	7·5	125
1937	51	43 (135)	7·2	127
1938	48	52 (233)	9·6	130
1939	41	36 (107)	10·5	152
1940	38	30 (142)	6·8	138
1941	50	54 (104)	7·0	120
1942 ($7\frac{1}{2}$ months)	41	42 (488)	9·0	136

In the first $7\frac{1}{2}$ months of 1942 the estimation of b has been made from more than twice as many values as in any previous year. Future variations of all important factors will be followed with correspondingly greater accuracy. The highest average error for any one year for ($P=0·95$), 3-weeks, 10-pairs, is 66 to 152 %, which is a little lower than the British Pharmacopoeia error for a 40-pair assay (63–159 %).

Finally, the records of assays carried out on the new plan have yielded information on the comparability of estimates of b from Standard and from test materials. Altogether there were 24 assays, involving 416 values, in which β-carotene Standard was compared with test-materials in which vitamin A itself supplied the whole of the vitamin A activity (Table 5).

Table 5. *Comparison of estimates of slope from response to β-carotene and to vitamin A*

	No. of values	Mean b	S.E.	Variance	Degrees of freedom n
β-Carotene (S)	221	43·6	2·69	1601	197
Vitamin A (T)	195	39·6	3·02	1781	171
Difference		4·0	4·05		

Variance ratio = 1·112. $z=\frac{1}{2}\log_e 1·112=0·0531$.
S.E. of $z=\sqrt{\{\frac{1}{2}(1/n_S+1/n_T)\}}=0·0739$.

Neither the two b's nor the variances are significantly different; thus an important condition of satisfactory assay work is fulfilled. This conclusion depends to some extent on the mean Standard: test-

material dose-ratio of the component assays, because in a series in which the mean dose-ratio is much higher or lower than unity a systematic difference due to departure from parallelism may invalidate the result. The mean dose-ratio of the above 24 assays is sufficiently close to unity, being approximately 1·1.

SUMMARY

(i) For the vitamin A-test rats in this laboratory interlitter variations in response are large enough to justify strict adherence to isogenic comparisons. The ratio of the numbers of isogenic or anisogenic pairs required for equal accuracy is about 1 : 2.

(ii) A new assay plan for routine work is described. A moving average estimate of b, the constant of slope, is employed.

(iii) Real variations in b are found to occur over comparatively short periods.

(iv) The b's for vitamin A and β-carotene are shown to be substantially similar.

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Further Studies of Cryptogam Tissue Proteins: Protein Composition and Tissue Function in Plants

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In an earlier article [Lugg, 1940] the amide and some amino-acid contents of the photosynthesizing tissues of *Lunularia cruciata*, *Pteridium aquilinum* and *Selaginella uncinata*,* and similar data for leaf protein of *Trifolium subterraneum*, were reported. It was pointed out that the amide, tyrosine and tryptophan contents revealed no marked changes in the compositions of the proteins to correspond with the different degrees of evolutionary development of the three cryptogams and of the phanerogams (i.e. *Trifolium subterraneum*) which have been examined.

In this article, the results of further analytical work with the same protein preparations will be reported. Some data are given for one of the extracted protein preparations from pinnae of *Pteridium aquilinum*, and the protein in the thalli of *Ulva lactuca*.

METHODS

The protein preparations. With the exception of the *Ulva lactuca* preparation, the protein preparations and the tissues from which they were obtained, have been described [Lugg, 1940].

The *U. lactuca* preparation was made from thalli collected on 11 April 1941 from a rocky ledge in the Southern Ocean near Port Elliot. The ledge was completely submerged at high water but merely washed over by the waves at low water. The thalli, thoroughly wet with sea water, were examined about 36 hr. after collection. They did not differ in general appearance from freshly collected material, nor appear more flaccid. They were rinsed with distilled water and pressed between sheets of blotting paper. After being weighed, they were dried at 65° in a current of air and then powdered, prior to removal of non-coagulable and other non-protein nitrogenous substances [Lugg, 1939]. Complete drying was effected by heating at 105° for about 8 hr. and exposing at low pressure over H_2SO_4. Nitrogen estimations were made by the Kjeldahl method.

The fresh thalli contained 15·8 % dry material consisting of 1·18, 0·90 and 0·83 % of 'total', 'coagulable' and 'protein' N respectively. The dried 'whole protein preparation' contained 2·04 % N, and 11·5 % ash (by ignition in air at about 600°). The ash contained large amounts of $CaSO_4$.

Partial analyses of the protein preparations. The

* Previously reported as '*Selaginella* sp. (probably Jungermannioides group)'. I am indebted to Prof. J. G. Wood for completing the identification of this plant. *S. uncinata* is not in the Jungermannioides group.

amide, tyrosine, diiodotyrosine, tryptophan, cystine plus cysteine and methionine contents of the protein preparations were estimated by methods described in earlier articles [Lugg, 1937; 1938 *a*, *b*, *c*]. The arginine, histidine and lysine contents of one of the extracted protein preparations from pinnae of *Pteridium aquilinum*, were estimated by Tristram's [1939] methods. From tests which have been recorded with the descriptions of these methods of estimation, it seems probable that the considerable impurities in the protein preparations would not have vitiated the results obtained (Table 1). Estimations by the other methods (Table 2) are vitiated to some extent by impurities such as carbohydrates, and sometimes by small amounts of prosthetic carbohydrate. In both tables the amino-acid contents have been corrected for such losses as the pure free amino-acids undergo in the procedures (including hydrolysis) employed in the estimations [Lugg, 1938 *a*, *b*; Tristram, 1939].

In Table 1 are shown, in the order of the columns, the characteristic label of the preparation consisting of a number followed by W, E or R, signifying respectively that the preparation was made from whole tissue, a tissue extract, or from tissue residues after extraction of some of the protein, followed by the name of the plant and tissue from which the preparation was derived, and the analytical data.

Most of the amide, tyrosine, diiodotyrosine and tryptophan contents have been already reported [Lugg, 1940], and the preparations are numbered as before. The *Ulva lactuca* preparation has the number 0. The numbers are in the order of what is customarily accepted as the increasing degree of evolutionary development of the plants.

Table 1. *Partial analysis of the plant-protein preparations by the method of Lugg* (*see p. 133*)

Preparation (see text)	Total N (%)	% total N: Amide	Tyr.	Diiodo-tyr.	Try.	Cyst.	Meth.	Cyst. + meth.	SO_4-S ×100/N	Ash
0W *Ulva lactuca* (thalli)	2·04	5·59	1·34	0·0	1·01	2·2	1·2	3·4	124·5	11·5
1W *Lunularia cruciata* (thalli)	3·92	5·52	2·43	0·0	1·70	1·31	1·36	2·67	0·51	3·5
2W *Pteridium aquilinum* (pinnae)	5·82	5·12	2·43	0·0	1·70	1·31	1·36	2·67	0·51	3·5
2E ,,	11·1	4·97	2·08	—	1·13	1·11	1·71	2·82	0·02	1·4
2R ,,	2·70	5·14	2·20	—	1·13	1·20	1·65	2·85	0·37	1·6
2E1 ,,	12·0	4·92	2·14	0·0	1·19	1·16	1·74	2·90	0·06	1·3
3W *Selaginella uncinata* (leaves and stems)	3·12	5·31	2·60	0·0	1·43	1·22	1·49	2·71	0·64	9·9
4W *Trifolium subterraneum* (leaves)	7·17	5·29	2·53	—	1·75	1·15	1·32	2·47	0·08	0·7
4E ,,	14·1	5·35	2·64	—	1·62	1·13	1·38	2·51	0·00	0·4
4R ,,	5·26	5·39	2·64	—	1·67	1·12	1·29	2·41	0·00	3·1

Estimations unaffected by presence of carbohydrate. Amino-acids are given as percentage of total N.

Percentage errors, from replicate estimations in present and earlier work; N in dry preparation 0·5, amide 1·5–2, tyrosine 2–3, tryptophane 3–4, cystine + cysteine 3–5, but 20 for 0W, methionine 4–6, but 30 for 0W; sum of last two 2–3, but 10 for 0W. The error for arginine, histidine and lysine estimations from the work of Tristram [1939].

Table 2. *Partial analysis of the protein preparations by other methods* (*see p. 133*)

	HI digestion and titration		Tristram [1939] (see p. 134)		
	Cyst.	Meth.	Arg.	Hist.	Lys.
2E1	1·03	1·80	13·5*	2·4	6·45
4E	0·93	1·16	—	—	—

Estimations affected by the presence of carbohydrate and not corrected for the effects.

Amino-acids are given as percentage of total N. Corrections were applied according to Tristram [1939]. Arginine +2·6 mg. N in addition to solubility correction of Ag. arginine. Histidine + 0·3 mg. N. Lysine + 1·6 mg. N. Hydrolysis factors 1·06, 1·03 and 1·07 respectively.

* This value increased to 15·3 when corrected for effects of presence of carbohydrate impurity.

DISCUSSION

From comparison of the partial compositions of 'whole', 'extracted' and 'residual' protein preparations (Table 1), the conclusion was drawn that 2E, 2E1 and 4E were reasonably representative of the 'whole' proteins in the tissues from which they were extracted, and the analyses shown in Table 2 are, therefore, assumed to be analyses of representative proteins, but they are undoubtedly subject to systematic errors, because such preparations contain impurities of a carbohydrate nature (e.g. pentosan or mucilage).

Lugg [1938*b*] found that these impurities affected the estimations of cyst(e)ine and methionine by the HI digestion procedure differently with different protein preparations and that the differences were

irregularly related to the amounts of impurity*, the standards of reference being estimations by the differential oxidation procedure. Thus corrections could not safely be attempted, and the results are only imperfect checks upon the values obtained by the less specific differential oxidation procedure. Note that the methionine content of 2E1 was actually slightly higher when estimated by the former procedure than by the latter. This may have been due to the presence of an impurity in the HI digest capable of reacting as methionine does in the titration procedure, but random errors could easily account for the discrepancy. Nearly all the cyst(e)ine plus methionine estimated by the differential oxidation procedure could be accounted for by the HI digestion procedure in this instance, which is unusual with preparations as impure as 2E1 appears to be (see below).

Tristram [1939] found that the influence of these impurities upon the estimations of histidine and lysine was irregularly related to the amount of impurity, but was small when the latter was not overwhelmingly large. For amounts of impurity such as those in 2E1, he considered the effects too small and uncertain to justify corrections. He found, however, in the estimation of arginine that the effects of small amounts of impurities were sufficiently regular to warrant the use of a correction factor calculable from the estimated percentage impurity.

Two methods of estimating the amount of impurity in preparations like 2E1, or of estimating the percentage N in the hypothetically 'pure' proteins, have been discussed elsewhere [Chibnall, 1939]. They are (1) computation from extensive amino-acid analyses of an impure preparation, and (2) calculation from the amount of humin precipitated at *p*H 2·5 after acid hydrolysis on the assumption that the impurity has behaved like a pentosan. These methods lead to substantially the same results when applied to extracted preparations from *Dactylis glomerata*. Since the leaf proteins of all phanerogams so far examined have very similar compositions, there is some justification for the assumption that the pure proteins all contain about 17 % N. The pinna protein of *Pteridium aquilinum* has not been sufficiently examined to permit the use of method (1) except in a very loose way, i.e. since from Table 1 it corresponds fairly closely in composition with the leaf proteins of the phanerogams examined, it too probably contains about 17 % N, if pure. Data for method (2) were obtained from both 2E and 2E1, which yielded respectively, 28·2 and 24·8% of 'humin', containing 7·4 and 6·9 % of the protein N.

* Smith & Wang [1941], ignoring these complications, have contended from their analyses of pasture protein preparations by this procedure, that the protein composition varies somewhat with season.

Calculations from these data (ignoring ash) suggest that the 'pure' preparations would have contained 17·8 and 17·4 % N respectively and it seems reasonable to accept the rounded value, 17 %. The actual preparation must have contained 30 % impurity, and from Tristram's [1939] correction table, the correction factor to be applied to the arginine content reported in Table 2 is 1·20/1·06. The corrected value is shown in a footnote to the table.

In Table 3 the partial composition of the proteins in the somatic photosynthesizing tissues of the cryptogams and those of various phanerogams are compared. The arginine estimations are corrected for impurities. The plants are grouped into families, orders, classes and divisions in the natural classification. The divisions have been arranged from left to right in the sequence of increasing evolutionary development, and the sequence has been maintained, as far as current evolutionary theory permits, down to the genera. Some of the extracted protein preparations are known not to have been fully representative of the tissue proteins, but they probably did not differ from them very much. These data have been drawn from material in the present article and from analyses by Lugg [1938*c*; 1939] and Tristram [1939]. Mean values are quoted where analyses have been made of more than one preparation from any particular plant species.

The striking feature of Table 3 is that among representatives of the bryophyte, pteridophyte and spermatophyte divisions, differences in tissue protein composition are small, the greatest variability being in the tryptophan content. The tissue protein of *Pteridium aquilinum* has a lower tyrosine and tryptophan content than those of the other plants belonging to these three divisions, and as compared with these its methionine content is rather high relative to its cyst(e)ine content; but in its amide, arginine, histidine and lysine content it is very similar to the others.

Unfortunately the cystine and methionine contents of the tissue protein of the only representative of the thallophyte division (*Ulva lactuca*) are subject to considerable errors, owing to the 2·5 % of sulphate-S in the preparation. This was present possibly as sulphuric acid esters of substances of carbohydrate nature. The possibility of the presence of nitrogenous substances other than protein, in preparations like 0W, cannot be ignored. However, the rate of production of NH_3 by hydrolysis (in the amide-N estimation) was very similar to those found for phanerogam leaf-proteins [Lugg, 1938*c*], and the presumption is that non-protein nitrogenous substances had been removed completely. The cystine content of the tissue protein is rather high and the tryptophan rather low, in comparison with those of the other proteins, and though there is nothing unusual in the amide content, the tyrosine

content is decidedly low. Many marine algae (notably members of the class Phaeophyceae) contain fairly large amounts of iodine compounds, but this *U. lactuca* preparation (like the other preparations examined) contains no diiodotyrosine.

bility than are revealed in Table 3. It is not known whether the composition of whole proteins of cryptogam tissues are as independent of climate, locality of growth, manurial treatment and tissue age, as are those of the phanerogams. Small varia-

Table 3. *Partial compositions of the proteins of the somatic, photosynthesizing tissues of plants*

Division	Thallophyte	Bryophyte	Pteridophyte		Spermatophyte				
Subdivision	Algae	Hepaticae			Angiospermae				
Class	Chlorophyceae				Monotocyledon		Dicotyledon		
Order	Ulvales	Marchantiales	Filicales	Lycopodiales	Glumiflorae		Centrospermae	Rosales	
Family	Ulvaceae				Gramineae		Chenopodiaceae	Leguminosae	
Genus and species	*Ulva lactuca*	*Lunularia cruciata*	*Pteridium aquilinum*	*Selaginella uncinata*	*Dactylis glomerata*	*Lolium perenne*	*Beta cicla*	*Trifolium subterraneum*	*Trifolium pratense*
Amide	5·59	5·52	5·12	5·31	5·07	5·22	5·28	5·39	*5·40*
Tyrosine	1·34	2·43	2·06	2·60	2·48	2·45	2·63	2·53	*2·50*
Diiodotyrosine	0·0	0·0	*0·0*	0·0	*0·0*	—	—	—	—
Tryptophan	1·01	1·70	1·14	1·43	1·84	1·84	1·50	1·75	*1·70*
Cyst(e)ine	2·2	1·31	1·14	1·22	1·40	1·44	1·45	1·15	*1·25*
Methionine	1·2	1·36	1·63	1·49	1·54	1·53	1·43	1·32	*1·29*
Arginine	—	—	*15·3*	—	*14·6*	*14·0*	*12·8*	—	*14·9*
Histidine	—	—	*2·4*	—	*2·4*	*2·4*	*2·25*	—	*2·3*
Lysine	—	—	*6·45*	—	*6·1*	*6·0*	*6·7*	—	*6·5*

Estimations corrected where necessary for carbohydrate impurities. Values in italics are for extracted protein preparations: those in ordinary type for whole protein preparations.

Amino-acids are given as percentage of total N.

Little work has been done with cryptogams other than unicellular organisms like yeasts and bacteria. Mazur & Clarke [1938] studied the amino-acid composition of the nitrogenous substances not extracted by alcohol from the tissues of various marine algae. In thalli from one specimen of *U. lactuca* they found that 5·5% of the remaining N was amide-N; and in this and another specimen, that 0·0 and 0·0% was tyrosine-N, 0·3 and 0·6% was tryptophan-N, 1·8 and 2·1% was cyst(e)ine-N, and 0·0 and 0·0% was methionine-N. Except for the amide and cyst(e)ine content, these results are very different from those reported in Table 1. Of the N remaining after alcohol extraction in tissues of *Osmunda claytoniana* (pteridophyte), the same authors found 0·6% cyst(e)ine-N, 1·1% methionine-N, 7·6% arginine-N, 3·7% histidine-N and 3·6% lysine-N. These results again differ considerably from the values in Table 1. Mazur & Clarke's analyses were actually analyses of the nitrogenous substances dissolved out by hot formic acid solution from the alcohol-extracted tissues, and it is not certain that the formic acid dissolved out unaltered protein.

The amounts of those amino-acids which were not estimated may exhibit different degrees of variations have been reported by Chibnall [1939], Lugg [1938 *a*, *b*; 1939] and Tristram [1939] for the whole proteins of the leaves and for preparations believed to be fairly representative, but in regard to age of tissue the sources of material included neither seedlings at the one extreme nor senescent plants at the other. Barrien & Wood [1939] and Hanson, Barrien & Wood [1941] have shown that the N : S ratios in their protein preparations from Sudan grass (*Andropogon sudanensis*) were higher for seedling leaves than for older leaves. Their S estimations, however, included any sulphate-S which may have been present. Reference has been made above to a claim by Smith & Wang [1941], of variation with season, but there is, as yet, no conclusive evidence that the composition of the whole leaf protein of any phanerogam varies markedly with changes in the conditions enumerated.

Phanerogam tissues contain at least two proteins of somewhat different composition. These have been tentatively referred to the fluid protoplasm and vacuole contents on the one hand, and to the granular inclusions like chloroplasts, chondriosomes and nuclei on the other [Lugg, 1938 *a*, *b*; 1939]; or to chloroplasts on the one hand, and the rest of the protein-containing morphological units on the

other [Barrien & Wood, 1939; Hanson *et al.* 1941]. Chibnall [1939] showed that vacuole protein may differ somewhat in composition from the proteins of the fluid and granular parts of the protoplasm.

In these somatic, photosynthesizing tissues two well-defined types of process occur—the ordinary living processes of the cell, and those processes intimately connected with photosynthesis—and it is possible that the physical and chemical properties and compositions of the proteins associated with these processes vary very little. Perhaps also the proportions of the different proteins differ little in different species, different climates, with different ages of tissue and so on.

It is generally agreed that present-day bryophytes, pteridophytes and spermatophytes represent increasing degrees of evolutionary development from a common ancestor; and there is no reason for supposing either that the basic living processes, or that the photosynthesizing processes, have changed radically, either in the gametophyte or the sporophyte generations of the plants. These considerations are in harmony with the observation recorded in this article, namely, that the 'whole' proteins of the gametophyte tissue of bryophytes, and of the sporophyte tissue of pteridophytes and spermatophytes, appear to be of similar composition. It is generally accepted that the Chlorophyceae (Thallophyte) are in the same main branch of evolutionary descent as the green land-plants [Bower, 1929; 1935; Fritsch, 1929]. It is likely that the *Ulva lactuca* thalli belong to both the sporophyte and the gametophyte generations of the plant [Föyn, 1929]. Possibly the individual proteins in the thalli of *U. lactuca* are similar to those of these other plants, but occur in very different proportions. The radical evolutionary step by which the tissues were transferred from a water to an air environment may have demanded modification of the composition and physical properties of individual proteins. It would be of interest to discover, from analyses of tissue proteins of sea phanerogams, whether the reverse step has required any modification of protein composition.

In sharp contrast to what appears to be the relative uniformity of composition of 'whole' proteins of the somatic photosynthesizing tissues of the bryophytes, pteridophytes and spermatophytes, is the non-uniformity of composition of proteins in tissues characteristic of the spermatophytes alone, namely, the seeds. Since the seedling depends for its early growth upon the seed proteins, these should correspond fairly closely in composition with the leaf proteins. This is sometimes not the case. True, satisfactory analyses have been made only of the proteins of angiosperm seeds, and these appear to be elaborated in cells possessing $3n$ chromosome nuclei, but in regard to tissue protein composition no particular significance can be attached at present to chromosome number (cf. tissue protein composition of *Lunularia cruciata* possessing n chromosome nuclei, with those of the pteridophytes and spermatophytes possessing $2n$ chromosome nuclei). Leaf proteins appear to be of 'fibrous' type and are very readily denatured, whereas many seed proteins are of 'globular' type and can withstand prolonged periods of partial desiccation at relatively high temperatures. Possibly biological fitness for survival might otherwise be assured by fairly close correspondence of composition, whereas denatured seed proteins may be of little use to young seedlings; resistance to denaturation of seed proteins may be almost a prime criterion of fitness at the present stage of evolutionary development of the spermatophytes, an unfavourable composition of the proteins from the point of view of seedling nutrients being in comparison a small disadvantage.

The comprehensive analysis of a single protein preparation is laborious, and there are many species of pasture plants. The object of the investigation here described was to extend, if possible, the basis of the belief that for the purposes of animal nutrition studies, comprehensive analyses of pasture proteins might well be confined in the first instance to those of only a few species representative of the most important families of pasture plants. It is considered that the investigation has substantially broadened the basis for that belief.

SUMMARY

Partial analyses have been made of the 'whole' proteins of the somatic, photosynthesizing tissues of a variety of cryptogams (a thallophyte, *Ulva lactuca*; a bryophyte, *Lunularia cruciata*; and two pteridophytes, *Pteridium aquilinum* and *Selaginella uncinata*), and also of one more phanerogam (*Trifolium subterraneum*).

The apparent similarity of composition of tissue proteins of plants from the bryophytes upward through divisions exhibiting increasing degrees of evolutionary development, has been contrasted with the pronounced lack of uniformity in the compositions of seed proteins. An attempt has been made to interpret the findings in terms of hypothetical relationships between tissue function and environment, and protein composition and physical and chemical properties.

The assistance of Mr R. A. Weller is gratefully acknowledged. Thanks are due also to Miss B. S. Barrien and Miss C. M. Eardley for the collection and classification of plant material and for helpful discussions.

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A Simple Fluorescence Comparator and its Application to the Determination of Porphyrin

By C. RIMINGTON, *With an addendum by* E. Schuster, *From the National Institute for Medical Research, London, N.W. 3*

(*Received 13 October 1942*)

Several substances of biochemical interest are either themselves fluorescent in radiation of suitable wavelength, or are readily transformed quantitatively into fluorescent materials. Among these may be mentioned the porphyrins, stercobilin and related pigments, flavins, including vitamin B_2, certain chemotherapeutic drugs and aneurin.

Quantitative methods based on the measurement of fluorescence intensity have the advantages of being highly specific and extremely sensitive, enabling very small quantities of material to be determined with accuracy once they are suitably extracted. Such methods suffer, however, from the disadvantage of requiring, for accurate results, relatively expensive apparatus such as the combination of an ultraviolet source with the Zeiss or Pulfrich photometer or some type of photoelectric colorimeter.

For the purposes of a particular investigation it became necessary to carry out a number of quantitative determinations of porphyrin in single specimens of urine, each containing about 2μg. of porphyrin. Only measurements of fluoresence intensity were sufficiently sensitive, and in order to bring the method within the reach of the small medical or factory clinic an inexpensive fluorimeter or fluorescence comparator was essential which, acting very much like an ordinary colorimeter, would give results of sufficient accuracy for general purposes. It was realized that, with suitable adjustments, the instrument would serve for the determination of a number of fluorescent materials in addition to porphyrins, e.g. vitamins, drugs etc., as noted above. Its construction, and operation with porphyrin solutions, will be described in the present paper, whilst other applications will be dealt with in subsequent communications.

Requirements of a fluorescence comparator

The essential requirements of a fluorescence comparator are:

(1) An easily operated source of ultraviolet radiation with suitable filter to remove visible light.

(2) An optical system to conduct the visible fluorescent light into the eyepiece. This should not be in the same axis as the ultraviolet source. The emergent fluorescence is collected in the present instrument at an angle of 55° to the ultraviolet beam and is reflected by a system of mirrors into the eyepiece, the optical path being approximately equal to the distance of normal vision, so that lenses may be dispensed with. A suitable filter is interposed, the transmission of which covers a suitably narrow band in the region in which the fluorescence lies. With porphyrins, Ilford spectral filter no. 607 (orange) transmitting 595–650 mμ is used.

(3) A device for altering the intensity of one beam. In the present instance glass screens made by photographing a standard neutral-tinted wedge are used, and by adopting a reciprocating arrangement, rotation of a graduated knob brings about a gradual, but uniform, darkening of the field emanating from the standard. Matching is performed as in a colorimeter.

The construction and mechanical arrangements of the apparatus are described in the Addendum by Dr E. Schuster who has been responsible for the detailed design and who has kindly constructed the first four models produced.

METHODS

Calibration and operation of the apparatus for the determination of porphyrin

The extraordinarily intense fluorescence of porphyrin solutions has been studied by a number of workers from Dhéré [1933] onwards, one of the most detailed of recent investigations being that of Fink & Hoerburger [1933] in which the effects of *p*H, salt concentration, temperature etc. were described. These authors showed that porphyrin isomers could be recognized by differences in their *p*H-fluorescence curves.

Fikentscher [1932] first described a method of porphyrin determination based upon the measurement of fluorescence intensity in a Zeiss step-photometer, and in his article will be found a consideration of the factors governing the accuracy of any such method. With concentrations of from 0 to 10 μg. porphyrin/ml. fluorescence intensity is proportional to concentration (other things such as incident illumination remaining constant), but above this limit quenching intervenes.

An attempt to reduce the cost of the necessary apparatus has been made by Stevens & Turner [1937] who describe a fluorescence method in which a carbon arc serves as the source of ultraviolet radiation, and the fluorescence elicited is measured by an electrical circuit containing two photo-cells.

A much more convenient and relatively inexpensive source of ultraviolet radiation is the 'Osira' mercury discharge lamp with Wood's glass bulb, which is incorporated in the present machine. As fluorescent standard, a solution of coproporphyrin in 5% HCl is employed in a sealed Monax glass test tube, the concentration being 2 μg./ml. This is made up from the tetramethyl ester as follows: A stock solution is first prepared by dissolving 1·085 mg. of solid coproporphyrin tetramethyl ester (equivalent to 1 mg. free coproporphyrin) in 5 ml. of glacial acetic acid, and adding 5% HCl (137 ml. conc. HCl to 1 l.) to a total volume of 100 ml. This stock solution contains 10 μg./ml. and should be kept in the dark in a stoppered bottle. The acetic acid it contains increases its stability. Solutions of lower concentration are prepared from it by dilution, as required, and in such a way that the final concentration of HCl is 0·25% (see later). Fikentscher reports that his standards (0·375–0·75 μg./ml. made up in 5% HCl without acetic acid) suffered no change in 6 weeks.

Attempts to find a stable fluorescent dye to serve as an artificial standard in place of porphyrin have not been successful. An ideal standard, in addition to possessing stability, should both absorb at the same wave-length in the ultraviolet, and fluoresce in the same spectral region, as does coproporphyrin. Several substances have been investigated, one of the most promising being resorufin (alkali salt), but the intensity of its fluorescence is such that in concentrations sufficient to match 2 μg. porphyrin/ml. quenching occurs. Safranine, the use of which was kindly suggested to me by Mr Jope of the Nuffield Laboratory, Oxford, also exhibits quenching at concentrations higher than about 30 mg./l., corresponding in fluorescence intensity to 0·3 μg. coproporphyrin/ml.

A coproporphyrin standard which has been in regular use in one apparatus in a sealed tube has shown no deterioration during nearly 3 months.

Monax glass test-tubes are used for both standard and unknown solutions. This glass does not fluoresce in ultraviolet radiation and is moderately transparent. Other suitable makes such as Hysil (for which suggestion I am indebted to Mr Jope) may also be employed.

To calibrate the instrument the standard 2 μg./ml. porphyrin solution in its sealed tube is placed in the right-hand side of the instrument and varying dilutions of porphyrin in similar test tubes in the left-hand side; sets of 5 or 10 matchings are then recorded for each dilution. With the instruments we have constructed a plot of the scale reading against the porphyrin concentration exhibits a linear relationship (see Fig. 1). Since accuracy of matching is greater at the lower intensities, it is best to dilute a more concentrated solution with 0·25% HCl until it contains about 0·25 μg./ml. which affords a convenient intensity of fluorescence. The range of the instrument may be taken as being from 0·05 to 0·5 μg. porphyrin/ml.

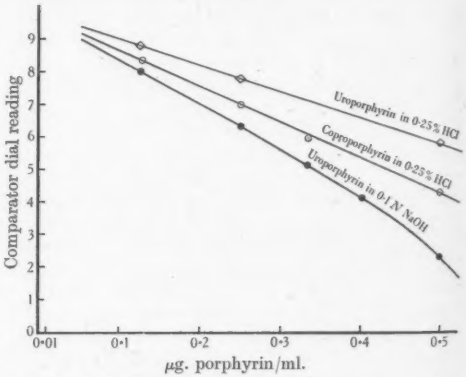

Fig. 1. Calibration curves of fluorescence comparator for porphyrin.

A porphyrin solution of unknown concentration is examined in exactly the same way, the mean of 5 or 10 matchings being accepted and the corresponding porphyrin concentration read off from the calibration graph.

As in all colorimetric work, it is advantageous to work in the dark. The sensitivity of the human eye to differences in colour intensity (especially reds) is known to vary considerably, but there is no doubt that any particular observer improves with practice.

Relation between pH and intensity of fluorescence

Fink & Hoerburger [1933] investigated the fluorescence of a variety of porphyrins both natural and synthetic, using buffer solutions to cover a *p*H range of from about 2·0–8·0, and found that, in general, fluorescence is minimal in the region of the isoelectric point, but increases progressively as the solution becomes more acid or more alkaline. An exception was found in the case of the aetioporphyrins which possess no carboxyl groups and fail to show the increase of fluorescence in alkaline solutions. The conclusion was drawn, therefore, that it is the + or − charged porphyrin molecule which is responsible for fluorescence.

As stated above Fink & Hoerburger's [1933] investigations did not extend to solutions more acid than *p*H 2 nor more alkaline than *p*H 8. Fikentscher [1932] had previously, and somewhat arbitrarily, selected 5% HCl as the medium to be used as solvent.

Using the fluorescence comparator described in this paper and a fixed standard, it was at once apparent that fluorescence in 5% HCl is much less than maximal and that small variations in acid concentration in this region result in relatively large discrepancies.

The *p*H fluorescence curves of coproporphyrin (I) and uroporphyrin (III) were explored from the isoelectric point to regions of high acid concentration as follows: Stock solutions of the porphyrins were made up from the esters in 5% HCl containing 5% acetic acid as previously described, the uroporphyrin concentration being 20 μg./ml. and the coproporphyrin 10 μg./ml. It may be noted here that 5% HCl is the most convenient strength of acid to use in preparing solutions from the esters; the pigment dissolves in this solvent in presence of acetic acid and hydrolysis of the ester takes place slowly on standing at room temperature. Na citrate-HCl buffers [Clark, 1928] to cover the range *p*H 4·95–1·17, whilst for still more acid solutions, unbuffered HCl of concentrations 0·5, 2·5, 5 and 10% were used. 0·1% acid afforded a useful comparison with the buffered solutions. 0·1 ml. quantities of the stock uroporphyrin or 0·2 ml. of coproporphyrin were diluted to 5 ml. (final concentration 0·4 μg./ml.) with the appropriate buffer, the *p*H of the solution being measured by the glass electrode and its fluorescence by the comparator. In the case of uroporphyrin (III) the investigation was extended to include the region on the alkaline side of the isoelectric point by means of Na citrate-NaOH buffers. The results are shown in Figs. 2 and 3.

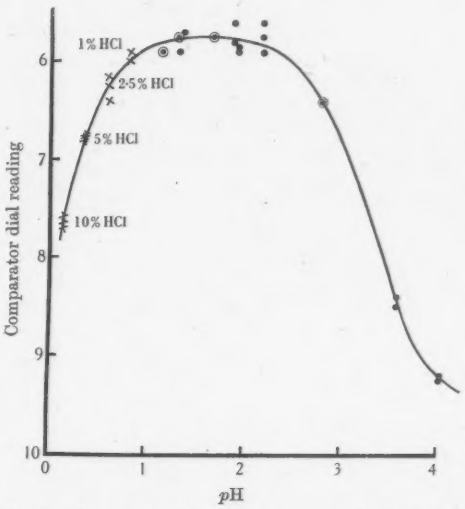

Fig. 2. Fluorescence intensity of coproporphyrin in acid solutions. Porphyrin concentration 0·4 μg./ml. • Buffered solutions. × Unbuffered solutions. ⊙ Coincident values.

The intensity of fluorescence of both porphyrins rises to a maximum at about *p*H 1·4, but declines rapidly as the concentration of acid is increased.

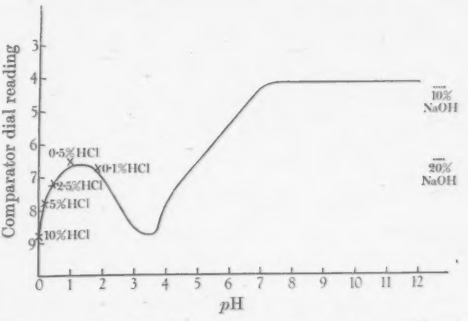

Fig. 3. Fluorescence intensity of uroporphyrin in acid and alkaline solutions. Porphyrin concentration 0·4 μg./ml.

5% HCl is seen to be a most unsuitable solvent for quantitative measurements, whereas in the region *p*H 1·2–2·0 for coproporphyrin, or 1·0–1·6 for uroporphyrin, fluorescence intensity remains practically constant. The acid number of coproporphyrin is fortunately low, viz. 0·1, so that a solvent such as 0·25% HCl having *p*H 1·24 can be used to extract it directly from ethereal solution.

On the alkaline side the fluorescence of uroporphyrin increases regularly from the isoelectric point to a *p*H of about 8·0 after which it remains constant until *p*H 12 (0·1 *N* NaOH) is reached. Even in *N* NaOH the intensity is substantially the same, but in 10 % NaOH it has dropped to about one-half, and in 50 % alkali it is practically extinguished.

In high concentrations of both alkali and acid, therefore, the intensity of porphyrin fluorescence is depressed. These changes are reversible, the porphyrins being relatively very stable molecules, and it seems reasonable to suggest on the basis of Fink & Hoerburger's [1933] theory that the fall in fluorescence intensity is due to suppression of the ionization of the molecule in presence of the overwhelming concentration of OH or H ions.

A practical outcome of these investigations is the suggestion that quantitative fluorescence intensity measurements for both uroporphyrin and coproporphyrin should be made in 0·25 % HCl (*p*H approx. 1·24) when small variations in *p*H will have relatively little effect upon the result. In the case of uroporphyrin it is convenient under certain circumstances to work in alkaline solution and for this purpose 0·1 *N* NaOH is a suitable solvent to employ. The fluorescence comparator has been calibrated for all these possibilities.

Equal quantities of uroporphyrin (I) and uroporphyrin (III) under the optimum conditions exhibit equal intensity of fluorescence. It should clearly be possible, however, to employ the instrument for identification of porphyrin isomers by plotting the *p*H-fluorescence curves according to Fink & Hoerburger in a series of buffer solutions on either side of the isoelectric point.

Preparation of the porphyrin extract from urine

In this paper the manipulation of the fluorescence comparator has been described first, since it is desired to emphasize that its function is to record the concentration of any given solution. Errors introduced in the preparation of the extract, variations in porphyrin excretion during the day etc., must not be confused with those inherent in the use of the comparator.

Little is known concerning the level of porphyrin excretion throughout the day, hence wherever possible a 24-hr. specimen should be collected and an aliquot portion of 25–50 ml. taken from this for analysis.* One-tenth of the volume of glacial acetic acid is then added, followed by 2 vol. of solvent prepared by mixing 9 vol. of ether with 1 vol. of light petroleum (B.P. 40–60°) and the mixture is shaken in a stoppered separatory funnel for about 1 min. The addition of petroleum minimizes the risk of emulsification whilst having no appreciable effect upon the extraction of the porphyrin. When separation is complete the lower layer is drawn off and discarded, and the ethereal layer is washed three times in succession with about an equal volume of distilled water. The porphyrin is now transferred to 0·25 % HCl by adding 2 ml. of acid, shaking and transferring carefully to a Monax test tube graduated at 5 and 10 ml., then repeating the extraction with 1·5 ml. and lastly with 1 ml. of acid. The pooled acid extracts are then made up to the 5 ml. mark, the tube stoppered and the contents mixed. It is then placed in the left-hand side of the fluorescence comparator and a set of readings made. Reference to the calibration graph gives the concentration of porphyrin/ml. of extract. Should the solution require further dilution, it may be made up to the 10 ml. mark with 0·25 % HCl. From start to finish a complete determination can be made in about 15 min. The used solvent may be collected for eventual recovery.

It will be noticed that only a single extraction of the urine with ether is performed. This is justifiable for a clinical method on grounds of speed, convenience, economy and the degree of complete extraction obtained. To perform a second extraction would mean the use of a second separatory funnel or an intermediary vessel to store the ether, and the use of twice the amount of solvent. Using solutions of pure coproporphyrin containing from 0·05 to 0·5 μg.

Table 1. *Comparison of porphyrin determinations on pathological urines by the standard method and the fluorescence comparator method*

Case	Diagnosis	Standard method μg. porphyrin/l.	Fluorescence comparator μg. porphyrin/l.	Difference %
Th.	Toxic jaundice	79·3	75	− 5·4
		89·7*		−18·5
Shep.	,,	319*	320	+ 0·3
Do.	Sprue	102	100	− 2·0
		25·2	25	− 0·8
Co.	Aplastic anaemia	70·8*	60	−15·3
Sh.	,,	111*	70	−36·9†
Sh.	,,	58*	60	+ 3·5
V.d.S.	Hepatic insufficiency	156	167	+ 7·1
		70	77	+10·0
Sm.	Hepatic cirrhosis	47·8	43	−10·0
Thom.	Addison's disease	77·7	77	− 0·9
Ber.	Acute idiop. porphyria	313	306	− 2·2
Ber.	,,	769	750	− 2·5
Ber.	,,	916	1080	+17·9
Res.	Pellagra	892·5	768	−13·9

* Determinations made by Dr J. Kench, Royal Infirmary, Manchester.

† The acid extract was much coloured by foreign pigment (from a highly pigmented urine) which probably led to a high spectrophotometric result.

* Mr Jope of the Nuffield Laboratory, Oxford, has informed me in a private communication that he finds the urinary porphyrin excretion may vary widely throughout the course of the 24 hr.

of porphyrin/ml., i.e. from approximately the normal to ten times the normal concentration in urine, and subjecting them to the extraction procedure outlined above, it has been found that the recovery is about 90%. Should turbidity affect the final acid extract, it may be centrifuged.

Agreement with the standard procedure

In order to test the reliability of the fluorescence method when applied to normal and pathological urines, a number of specimens were analysed by this technique and also by the standard procedure of Fischer, in which to 1 l. or more of urine, one-tenth the volume of acetic acid is added and the mixture then shaken twice with at least an equal quantity of ether. After washing repeatedly with water, the ether layer is shaken with successive quantities of 5% HCl and the porphyrin in the combined extracts determined spectrophotometrically (Hilger-Nutting apparatus calibrated by means of standard coproporphyrin solutions, readings being taken at a wave-length of 546–549 mμ in the centre of the β band of the acid absorption spectrum). Frequently, in some pathological urines, the initial acid extract contained brownish pigments without characteristic absorption spectra, which led to erroneously high porphyrin figures, but by careful repetition of the purification over ether and re-extraction with acid [Rimington & Hemmings, 1939] these could usually be eliminated and a reliable result be obtained. Through the kindness of Dr J. E. Kench of the Manchester Royal Infirmary many of the standard analyses were performed in Manchester, whilst the corresponding fluorescence measurements were made at Hampstead. Table 1 presents the results obtained.

ADDENDUM. By E. SCHUSTER

Construction of the apparatus

The apparatus is shown semi-diagrammatically in Figs. 4 and 5 which give respectively a sectional elevation and a sectional plan. It consists of a metal box, the back wall of which (1) is raised upwards for 4 in. and then bent backwards for 2 in. to form a flange (2) which coincides in plan with a backward prolongation of the base (3). Two vertical brass rods (4) connect the base with the flange, and a horizontal bracket (5) is clamped to these rods at an adjustable level. The lampholder (6) for the Osira lamp (7) is screwed to the under-surface of the bracket. Piercing the top of the box near the back wall are two guide tubes, of which the right-hand one receives the test tube (8) containing the standard solution and the left-hand one the sample (9). The bottoms of the test tubes are supported by sockets (10) attached to the base. Ultraviolet rays reach a section of each test tube through windows (11) in the back wall. Small brackets surrounding these windows serve to carry extra pieces of Wood's glass, for use in determinations in which a spectral filter is omitted. They eliminate the last traces of

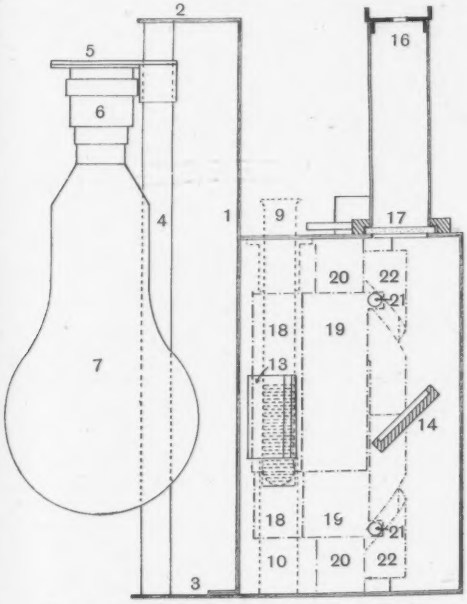

Fig. 4. Semi-diagrammatic sectional elevation (1/3 natural size).

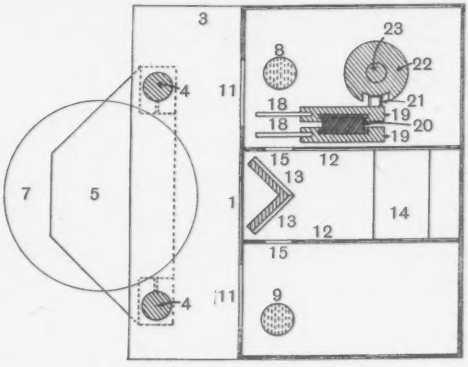

Fig. 5. Semi-diagrammatic sectional plan (1/3 natural size).

visible light from the source. Vertical partitions (12) separate off a central compartment of the box and serve as supports for the three mirrors which it contains. Two of these (13) lie at right angles to each other in vertical planes, while the third (14) makes an angle of 45° with the horizontal.

Fluorescent light from the two test tubes enters the central compartment through square apertures (15); it is reflected by the vertical mirrors on to the oblique mirror and thence upwards into the eyepiece (16) through a hole in the top of the box. The base of the eyepiece tube contains a filter (17) which cuts off practically all light except the wave-lengths produced by the fluorescence of porphyrin.

Interposed between the standard and the aperture (15) are the two glass screens (18); the dark end of one is above and of the other below, so that where they overlap they produce a uniformly shaded field. The screens are mounted in slides (19) which can move up or down a vertical guide (20). Each is held at the desired level by a pin (21) which enters a spiral groove in a vertical cylinder (22). The spiral is in one case right-handed and in the other left-handed. The two cylinders are fixed one above the other to a spindle (23) carried in bearings in the top and base of the box; a knurled head and a dial divided into a hundred divisions are mounted on the spindle above the top of the box. The spiral groove is of such a pitch that the whole range of movement of the slides is achieved by slightly less than one complete revolution of the spindle, and, as the grooves are right- and left-handed, equal and opposite movements are given to the two slides by a given degree of rotation.

The slides are of such a length that their less dense ends are separated by a gap of about $\frac{1}{4}$ in. when the dial knob is at zero, so permitting unobstructed light to pass in this position from the right-hand tube to the mirror for comparison with the tube on the left. This arrangement has been found convenient for certain determinations in which a blank had to be evaluated.

The inside metal work of the apparatus is painted black and the tube of the eyepiece is lined internally with matt surfaced black paper.

Thanks are due to Mr J. Smiles and his assistants of this Institute for kindly preparing the glass screens used in the construction of the instrument. I also wish to acknowledge the benefit of friendly discussions with my colleagues, Dr J. E. Kench of the Royal Infirmary, Manchester and Mr E. M. Jope of the Radcliffe Infirmary, Oxford. Dr Z. A. Leitner kindly secured some of the pathological urines used for test studies.

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The Utilization of Urea in the Bovine Rumen. 1. Methods of Analysis of the Rumen Ingesta and Preliminary Experiments *in vivo*

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Some evidence has accumulated during the past 40 years to suggest that ruminants can utilize simple non-protein nitrogenous compounds such as urea when these compounds replace a proportion of the protein normally present in the diet. The work, much of which is highly controversial, has been reviewed recently by Krebs [1937], and also by Owen [1941]. Since these reviews appeared, publications by Owen, Smith & Wright [1941; 1943] in this country and by Hart, Bohstedt, Deobald & Wegner [1939] in America have shown that urea undoubtedly has some definite nutritive value when fed to ruminants in suitable amounts and in appropriate mixtures. It has been suggested that certain micro-organisms which multiply in the rumen build up their own body protein from this simple form of nitrogen. These micro-organisms then pass further along the alimentary tract where their protein is digested with the ordinary protein of the diet. The work here described was undertaken to test the validity of this theory.

A steer with a large rumen fistula was employed and in the original plans, made in 1938, it was hoped to use this animal, in the first place for *in*

vivo experiments in which urea would be fed and any changes occurring in the N distribution in the rumen ingesta would be observed, and secondly for obtaining samples of the rumen contents for *in vitro* incubations with urea.

It was first necessary to establish reliable methods for the accurate determination of non-protein nitrogen (N.P.N.), urea and NH_3 in rumen ingesta, and also to study the heterogeneous nature of the rumen contents themselves, for it soon became clear that in any experiments carried out *in vivo*, sampling of the rumen contents would present great difficulties. The first paper of this series is therefore concerned with the details of the methods adopted for estimating N.P.N., urea and NH_3, with the heterogeneity of the rumen ingesta of the steer, and with some preliminary *in vivo* experiments.

METHODS

The estimation of N.P.N. in rumen contents

(*a*) *Rumen liquor.* The three protein precipitants which seemed most suitable were trichloroacetic acid, sodium tungstate in H_2SO_4 and alcohol. It is well known that for many substances the estimation of N.P.N. does not result in an absolute value but that different values are obtained by different processes and by slightly different applications of the same process. Tests with rumen liquor were therefore carried out to find how suitable these three precipitants were for the present investigations, and under what conditions they gave reliable values which could be readily duplicated. The sample of liquid rumen contents used contained, after straining through muslin, 2·1% total solids and 80 mg. total N/100 g. The experiments with this sample were planned to study the effect on the N.P.N. value of (1) the concentration of the precipitant, (2) the concentration of the rumen contents in the precipitant, and (3) the time for which precipitation was allowed to continue before filtration.

Precipitation with trichloroacetic acid. Approximately 5 g. of the rumen liquor were accurately weighed into each of a series of flasks and made up to 100 ml. with $CCl_3.COOH$ solution of various concentrations ranging from 2·5 to 20%. The mixtures were thoroughly shaken and then filtered after 24 hr. At the same time further samples of the same rumen liquor were precipitated with 10% $CCl_3.COOH$, but the proportion of the rumen liquor to the total volume of the mixture varied from 1 in 5 to 1 in 50. Again filtration was performed after 24 hr. Finally mixtures were made up consisting of 1 part rumen liquor to 20 parts 10% $CCl_3.COOH$ and portions filtered after 1, 4 and 24 hr. The results are summarized in Table 1.

It appears from these results that with $CCl_3.COOH$ the time which elapses between precipitation and filtration is not of great importance. Up to 4 hr. there was little difference, and after 24 hr. the N.P.N. values had only increased by about 5%. Provided therefore that a definite time be chosen for all determinations, consistent results should be obtained. Decreasing the proportion of rumen contents to $CCl_3.COOH$ solution from 1 in 5 to 1 in 50 increased the N.P.N. value from 24·5 to 28·2, but note that the values obtained for a ratio of 1 in 5 and 1 in 10 differed only very slightly and that the same was true for the two values obtained with ratios of 1 in 20 and 1 in 50. Consistent results should therefore be obtained with this precipitant by keeping the proportion of rumen liquor to $CCl_3.COOH$ approximately constant throughout. The actual concentration of $CCl_3.COOH$ is obviously important, for the higher the concentration the greater is the N.P.N. value. Throughout the present work a 10% solution was used.

Precipitation with sodium tungstate in sulphuric acid. 50 ml. 10% sodium tungstate solution were added to 10 g. of rumen liquor and the mixture made up to 500 ml. with H_2SO_4 of various strengths. It was filtered after 24 hr. The effect of varying the proportion of rumen liquor to precipitant and of varying the interval before filtration was also studied.

Table 1. *Estimation of N.P.N. using trichloroacetic acid as precipitating agent*

(mg./100 g. rumen liquor)

Conc. of trichloroacetic acid (%) (1 part rumen liquor to 20 parts total mixture; filtered after 24 hr.)	N.P.N.	Ratio of rumen liquor to total mixture; made up with 10% trichloroacetic acid (filtered after 24 hr.)	N.P.N.	Time of filtration (hr. after precipitation), 1 part of rumen liquor in 20 parts of total mixture; made up with 10% trichloroacetic acid	N.P.N.
2·5	22·1	1 in 5	24·5	1	25·2
5·0	23·9	1 in 10	25·2	4	25·0
7·0	25·3	1 in 20	27·3	24	26·6
10·0	27·2	1 in 50	28·2		
15·0	33·3				
20·0	35·7				

The results (Table 2) show that when filtration was carried out 24 hr. after precipitation, all concentrations of H_2SO_4 between *N* and *N*/10 tended to give very similar results, whereas with more dilute acid the values were much higher. These high values were probably due to incomplete precipitation, for it was difficult to obtain a clear filtrate at the greater dilutions. The same explanation probably holds for the higher values obtained with *N*/6 acid when filtration occurred 1 or 4 hr. after precipitation. Table 2 also shows that the ratio of rumen liquor to total volume of mixture has little effect on the N.P.N. value provided 24 hr. elapse before filtration.

Precipitation with alcohol. Similar tests were made using alcohol as the precipitant. The results (Table 3) show that the value for N.P.N. increases as the concentration of alcohol diminishes, due undoubtedly to less complete precipitation as the water content of the alcohol increased, since with 96% alcohol a clear filtrate could be obtained almost immediately, whereas with the 60 and 40% alcohols filtration was slow and tended to give turbid filtrates. With 96% alcohol the N.P.N. values were little affected either by the ratio of rumen liquor to alcohol or by the time between precipitation and filtration.

Table 2. *Estimation of N.P.N. using sodium tungstate and H_2SO_4 as precipitating agent*

(mg./100 g. rumen liquor)

Normality of H_2SO_4 (1 part rumen liquor made up to 50 parts total mixture; filtered after 24 hr.)	N.P.N.	Ratio of rumen liquor to total mixture made up with $N/6$ H_2SO_4	N.P.N. filtered after 1 hr.	4 hr.	24 hr.
N	18·8	1 in 5	21·2	20·9	20·5
$N/2$	18·0	1 in 10	21·5	22·2	19·6
$N/6$	17·6	1 in 20	23·6	24·2	18·5
$N/10$	18·2	1 in 50	27·4	27·6	18·0
$N/12$	24·1	1 in 100	33·6	33·1	19·0
$N/16$	24·0				
$N/20$	26·4				

Table 3. *Estimation of N.P.N. using alcohol as a precipitating agent*

(mg./100 g. rumen liquor)

Conc. of alcohol (% by vol.) 1 part of rumen liquor to 20 parts total mixture; filtered after 24 hr.	N.P.N.	Ratio of rumen liquor to total mixture made up with 96% alcohol	N.P.N. filtered after 1 hr.	4 hr.	24 hr.
96	18·3	1 in 5	18·5	18·5	19·2
80	19·5	1 in 10	17·7	18·7	18·3
60	33·1	1 in 20	16·3	16·3	17·7
40	40·3	1 in 50	16·4	17·7	17·6

It is obvious from these first three tables that when filtration was carried out after 24 hr. the 96% alcohol and sodium tungstate methods gave similar results (17·6–19·2 and 18·0–20·5 mg./100 g. respectively), whereas 10% CCl_3.COOH (1 part rumen liquor to 20 parts total mixture) gave much higher values (26·6–27·3 mg./100 g.) although the same sample of rumen liquor was used for all the results recorded in Tables 1–3. Differences of this order (7–10 mg.) between values found by the alcohol and the tungstate methods and the value by CCl_3.COOH have always been observed when the three precipitants have been compared.

Of the three methods investigated the alcohol procedure is the simplest, but it was observed that if phosphate were added to rumen contents as a buffer when urea or NH_3 was present, the alcohol was liable to precipitate a portion of the NH_3, probably as magnesium ammonium phosphate. The alcohol procedure was therefore of very doubtful value as a routine method. CCl_3.COOH was the next choice in order of simplicity. This substance, however, is expensive and large quantities are not readily obtained in war time. Moreover, owing to the inactivation of urease by CCl_3.COOH the estimation of urea in such a filtrate presents difficulties. It was therefore decided to use sodium tungstate and H_2SO_4 as a general routine procedure, confirming the results periodically by parallel tests with CCl_3.COOH.

The method finally adopted was as follows. Approximately 10 ml. rumen liquor, accurately weighed, were added to 50 ml. $N/6$ H_2SO_4 in a 100 ml. flask and the mixture transferred to a 500 ml. volumetric flask with $N/6$ H_2SO_4; 50 ml. 10% sodium tungstate were added and the volume was made up to the mark with $N/6$ H_2SO_4. After standing for at least 24 hr. the mixture was filtered and aliquots of the filtrate were taken for analysis. Various trial experiments showed that the rumen liquor could remain in the 100 ml. flask with H_2SO_4 for at least 2 days before it was made up to 500 ml.; after that, any time between 24 and 48 hr. could elapse before filtration took place without any measurable change in the analytical results.

(*b*) *Solid rumen ingesta.* A sample of the solid rumen ingesta was taken from the rumen at the same time as the sample of the rumen liquor used in the experiments just described. It contained 14·3% of total solids and 290 mg. total N/100 g. The N.P.N. value estimated by 10% CCl_3.COOH was 40·2 mg./100 g., while the corresponding value by the sodium tungstate method was 19·6 and by the 96% alcohol method 20·3. With the solid ingesta therefore, as with the liquor, the alcohol and tungstate methods gave similar results, whereas the figure obtained by using CCl_3.COOH was much higher. Moreover, with the alcohol and tungstate methods the N.P.N. content of the solid ingesta was almost the same as that of the liquid (19–20 mg./100 g.), whereas by the CCl_3.COOH procedure the two values were very different, 40·2 mg./100 g. for the solid and 27·2 for the liquid. The solid and liquid ingesta might be expected to have similar N.P.N. values when estimated on the wet weight, since the solid material, which contains some 86% water, is constantly coming into contact with the liquid and so is likely to be impregnated with it. For this reason, and from these results, the authors believed that in routine work with mixtures of liquid and solid ingesta more accurate and consistent values for N.P.N. would be obtained by the tungstate method than by the use of CCl_3.COOH. The reason for the higher values obtained with CCl_3.COOH is not yet known.

The estimation of ammonia. Aliquots of the filtrate from the tungstate precipitation were neutralized with *N* NaOH using phenol red as indicator. 2 ml. *N* NaOH were then added in excess and the mixture was distilled for 10–15 min. in a current of steam, the distillate being collected in *N*/50 acid. This procedure gave results identical with those obtained when the acid aliquot was rendered alkaline by addition of solid MgO.

The estimation of urea. Aliquots of the tungstate filtrate were neutralized as in the estimation of NH_3, 10 ml. 0·6% KH_2PO_4 were added as a buffer, followed by 5 ml. of a 30% alcohol urease extract [see Peters & Van Slyke, 1932]. The mixture was incubated at 40–45° for 20 min. 2 ml. *N* NaOH were then added and the mixture was distilled in a current of steam for 10–15 min. The distillate was collected in *N*/50 acid.

In both the NH_3 and urea estimations blank determinations were made. The accuracy of the method was tested by determining urea in liquid rumen contents to which urea had been added in varying amounts. The results recorded in Table 4

Table 4. *Estimation of urea using tungstate as precipitating agent*

	(mg. N/100 g.)			
Urea-N added	1·52	3·04	6·08	7·60
Urea-N found	1·44	2·93	6·13	7·54
Recovery (%)	95	96	101	99
Urea-N added	15·2	30·4	60·8	152·0
Urea-N found	15·7	30·2	60·2	151·0
Recovery (%)	103	99	99	99

show that with less than 6 mg. urea-N/100 g. rumen contents the accuracy is about 95%, but that above that value the accuracy closely approaches 100%.

The heterogeneous nature of the rumen ingesta

The bovine rumen is an enlargement of the oesophagus and may contain as much as 200 lb. of material. The upper portions of the ingesta have a consistency similar to that of horse faeces, but as the rumen is descended the mass becomes increasingly moist, until 12–18 in. below the fistula the solid contents are bathed in fluid. At frequent intervals the whole mass of liquid and solid is mixed together by movements of the rumen musculatory system. A sample truly representative of this heterogeneous mass at any given time is difficult to obtain. Determinations of N distribution values on samples taken at the same time from different positions in the rumen showed a variation between extremes of almost 20%, and marked differences were also observed when samples of the material were taken from different depths in the rumen. In Table 5, the total N and N.P.N. values are recorded for moist but solid samples taken from different levels of the rumen. The N.P.N. values in particular show very wide variations. At depths below 8–12 in. the sampling difficulties become greater, for at these levels the proportion of liquid to solid withdrawn can be varied at will by the sampler. The sampling difficulties apparent in the results recorded in Table 5 are therefore much greater for samples taken from lower levels in the rumen where (below 8 in.) the greater part of the rumen contents is to be found.

Table 5. *Concentration of total nitrogen and N.P.N. of rumen ingesta from different depths of the rumen*

Depth below surface of ingesta in.	Water %	Fresh rumen contents (mg./100 g.) Total N	Fresh rumen contents (mg./100 g.) N.P.N.	Dry matter (mg./100 g.) Total N	Dry matter (mg./100 g.) N.P.N.
1	82·5	342	15·8	1949	90
4	84·3	321	20·1	2044	128
6	85·1	307	11·9	2053	80
8	83·9	288	12·3	1788	77
% difference between extremes	3·1	15·8	40·8	12·9	39·8

One of the aims of these experiments was to feed urea in a typical basal diet and study the changes in the rumen ingesta at intervals after the meal. Preliminary tests were therefore made to detect any variations in the total N of the rumen ingesta throughout the 10 hr. following a urea-free meal. Each sample analysed was a mixture of samples taken from four different parts of the rumen (see Table 6). It will be seen that total N values varied

Table 6. *Nitrogen content of rumen ingesta at hourly intervals after meal*

Time after meal hr.	Moisture %	Total N (mg./100 g.) Fresh rumen contents	Total N (mg./100 g.) Dry matter
1	80·0	360	1800
2	80·2	322	1628
3	80·5	362	1856
4	82·4	302	1716
5	82·0	311	1728
6	81·9	316	1746
7	81·6	322	1750
8	81·7	360	1967
9	81·9	328	1817
10	82·0	312	1733

very irregularly from 311 to 362 mg./100 g. for wet rumen contents, and from 1628 to 1967 mg./100 g. for dry matter. The authors believe that these variations were due mainly to sampling difficulties which made it impossible to detect with certainty the conversion of urea to protein in the rumen after a urea meal unless the amount of conversion were much greater than could be expected.

It was originally intended that extensive *in vivo* experiments should be done. The N distribution in the rumen on a diet containing no added urea was to have been determined. Urea was then to be included and its conversion into protein closely observed. After preliminary experiments, however, it appeared that sampling difficulties made precise

information as to the N distribution throughout the whole of the rumen ingesta at any given time unobtainable. Without such information any attempt to follow the conversion of urea to protein in the intact rumen was of little value.

Another difficulty also arises in interpreting the results obtained in *in vivo* experiments, because portions of the rumen contents are periodically passing from the rumen and reticulum into the other stomachs, and the material passing on at any time may not be representative of the rumen contents as a whole. It follows that if the small amount of urea or ammonia N present in the rumen immediately after a urea meal decreased within the next few hours, the decrease might be partly due to the passing of water-soluble urea or NH_3 into the other stomachs and intestines, and not necessarily to their conversion to protein. Even a simultaneous increase in the amount of total N in the dry matter of the rumen contents might be due to a selective passing-on of water-soluble carbohydrates and other constituents of the diet. These difficulties are illustrated by the results of an experiment in the *in vivo* section of the general investigations.

For 6 months the steer had received daily a mixture consisting of 2¾ lb. oats, 2 lb. bran, 1 lb. starch, ⅔ lb. molasses, 40 g. urea, 14 lb. hay and water *ad lib.* During this particular experiment the concentrate mixture was given in two portions, one at 9.45 a.m., the other at 4.30 p.m. Samples of rumen contents were removed at 9.30 a.m., 10.30 a.m., 12.30 p.m. and 3.30 p.m. The 9.30 samples should therefore have been representative of the rumen ingesta after a fast of 17 hr., the others of the material at varying periods immediately after the meal. At each sampling period semi-solid samples of approximately 600 g. were collected from near the top at each of the four 'corners' of the rumen and a fifth more liquid sample from deep down in the centre of the rumen. Without further treatment these five samples were analysed immediately in duplicate for water, total N and N.P.N. contents. Three days and also 6 days later this procedure was repeated. Thus, while the steer was receiving the basic diet plus urea, 96 values were obtained for the content of both the total N and the N.P.N. in the semi-solid ingesta, and 24 values for both these constituents in the liquid ingesta. Urea was then omitted from the basic diet and 9, 12 and 15 days later, the sampling and analytical procedure were repeated. The detailed results are too numerous to publish in their entirety. It appeared, however, that with both the semi-solid and the more liquid samples duplicate determinations for the same sample frequently differed by 20% or more. A similar difference was often observed in the values obtained for samples taken at the same time but from different parts of the rumen. Such large divergencies in individual values were too frequent to permit conclusions to be drawn as to the synthesis of protein from urea in the rumen.

The average results (Table 7) gave no evidence either for or against the theory that urea is utilized in this way. A study of Table 7 shows the following points:

(1) The concentrations of crude protein, true protein and N.P.N. were higher in the dry matter of the liquid ingesta than in that of the semi-solid material. In any quantitative experiment therefore the proportion of solid to liquid ingesta, and in fact the total amounts of each in the rumen, would have to be known.

(2) There was no significant difference between the protein contents of the rumen ingesta when the diet was fed alone and when it included urea.

(3) The only significant changes on the urea diet were in the N.P.N. fraction, e.g. 144 mg./100 g. in the dry matter of the liquid ingesta just before a meal had increased to 313 mg./100 g. 30 min. after the meal and had fallen again to 119 mg./100 g. some 5½ hr. later; the corresponding values for the semi-solid ingesta were 74, 118 and 59 mg. N/100 g.

Table 7. *Three-day average values for the content of crude protein, true protein and N.P.N. in the rumen ingesta on a diet containing urea and on the same diet without urea*

Time after previous meal hr.	Crude protein % dry matter		True protein % dry matter		N.P.N. mg. N/100 g. dry matter	
	Urea	No urea	Urea	No urea	Urea	No urea
	Semi-solid ingesta					
17	11·6	10·6	11·1	10·2	74	62
0·5	10·1	10·3	9·4	9·9	118	68
3	10·2	10·1	9·7	9·8	83	44
6	11·0	10·7	10·6	10·3	59	58
	Liquid ingesta					
17	15·1	17·2	14·2	16·7	144	83
0·5	17·3	15·6	15·3	14·8	313	130
3	16·3	14·9	14·6	14·1	276	128
6	15·3	15·8	14·6	14·9	119	139
Average of 96 duplicate determinations involving 48 samples of semi-solid ingesta	10·7	10·4	10·2	10·0	84	58
Average of 24 determinations involving 12 samples of liquid ingesta	16·0	15·9	14·7	15·1	213	120

Crude protein = 6·25 × total N; true protein = 6·25 × (total N − N.P.N.).

To show that urea was converted to protein in the rumen such an experiment should yield results such that the decrease in N.P.N. following the urea meal could be correlated with an increase in the 'true protein'. But here, if the fall in N.P.N. were entirely due to protein synthesis, a decrease in N.P.N. from 118 to 59 mg. N/100 g. and from 313 to 119 would mean an increase in true protein of only 0·4 and 1·2% respectively. The value of 9·3% for the true protein of the semi-solid ingesta 30 min. after the meal (Table 7) would therefore have to increase by 0·4–9·7%, some hours later, an increase which would not be significant owing to sampling errors. Similarly, the value of 15·3% for the liquid ingesta would have to increase to 16·7%. Actually in the solid material the value rose to 9·7% after 3 hr. and 10·6% after 6 hr., but in the liquid ingesta it decreased to 14·5%. These results therefore supply no proof of protein synthesis from urea. They are, however, not at variance with the suggestion that protein synthesis from urea did take place, because throughout the day the animal continued to drink at intervals and to secrete the usual large quantities of saliva. Moreover, part of the ingesta, particularly in the more liquid form, would be continually passing from the rumen. These processes would all tend to induce a diminution in protein and in N.P.N. content of the liquid ingesta as the interval after the meal increased. Simultaneously the semi-solid rumen ingesta might become slightly richer in protein because the more soluble carbohydrate material was washed out of the animal's food. By increasing the urea in the diet during the urea period the problem would not be solved but made more difficult, as the greater urea consumption would increase the water ingested and this would enhance the protein content of the solid ingesta and decrease that of the liquid.

DISCUSSION

The detection of urea conversion to protein by an *in vivo* experiment of this type is extremely difficult and seems never likely to yield results completely beyond dispute. Thus it seemed wise, at least in the early stages of the work, to concentrate on incubation experiments carried out *in vitro* when these difficulties would not arise, and to postpone the *in vivo* section of the general investigations for the time being. The main portion of the work completed therefore consists almost entirely of the *in vitro* experiments to be discussed in the next two papers of this series.

Since the original *in vivo* programme was temporarily abandoned, Wegner, Booth, Bohstedt & Hart [1941] have published a description of *in vivo* experiments using a heifer with a rumen fistula. The values for N.P.N. and total N of the rumen contents at intervals after the ingestion of a basal diet were compared with the corresponding values obtained when the diet included urea. Wegner *et al.* found that in general the crude protein content of the rumen ingesta was higher with added urea than it was on the basal diet alone, and that the N.P.N. of the ingesta fell to the basal level during the first few hours after a meal. They therefore suggested that urea utilization takes place in the rumen and that it occurs within 4–6 hr. after ingestion. An examination of the results, however, shows that such an interpretation is not necessarily correct, and that these workers have evidently encountered those very difficulties which the present authors anticipated. Thus from their 1st experiment it can be calculated that on the basal diet alone the true protein of the rumen ingesta appeared to increase from 8·4% at 1 hr. after a meal to 11·3% 4 hr. later, a difference of 2·9%. This was accompanied by a decrease in N.P.N. equivalent to only 1·0% true protein. When, however, the diet included urea, the corresponding increase in true protein was only 0·9% (9·9–10·8%), and this was accompanied by a decrease in N.P.N. equivalent to 1·5% protein. There therefore appears to have been less synthesis of protein with urea than with the basal diet alone during the 5 hr. following the meal. Again, the value of 11·3% for the true protein 5 hr. after the basal diet was fed, was actually slightly higher than 10·8%, the corresponding value when urea was included in the diet, a condition reversed 4 hr. later without any significant change in N.P.N.

In their 2nd experiment Wegner *et al.* [1941] fed a very abnormal basal diet consisting of 15 lb. corn silage and 3 lb. starch, following this by a period on the same basal diet with the addition of urea amounting to 5% of the dry matter of the ration. They found that of several diets which they tested, this most unusual one, which must have contained only about 5 lb. dry matter, gave the most marked results. Their values show that on two of the days when the basal ration was fed alone, the crude protein content of the rumen ingesta increased by 0·45 and 0·85% between either 1 or 2 and 8 hr. after the meal, but that on the only other day for which the corresponding values are available it decreased by 1·7%. No conclusion can be reached therefore as to whether the crude protein content of the rumen ingesta should be expected to rise or fall during the first 8 hr. after the ingestion of the basal diet. On the 2 days for which the corresponding values are given for the basal diet + urea, the crude protein decreased by 0·7% between 2 and 8 hr. after feeding on the first day, and remained unchanged on the second. Even correcting the crude protein for NH_3 where possible (N.P.N. values are not given), the maximum increase in protein following the ingestion of urea was only 0·6%. In relation to the changes occurring on the

basal diet alone, the results do not support the view that urea is converted to protein in the rumen during the first 8 hr. following a meal.

The evidence upon which Wegner *et al.* [1941] appear to place most confidence is that on the basal diet alone the crude protein content of the rumen ingesta varied from 8·9 to 10·6%, whereas with the addition of urea to the diet this value became much higher and varied between 12·0 and 12·7%. In all probability, however, these values might be due to the fact that when 100 g. urea were added to the diet which supplied only 5 or 6 lb. dry matter per day, of which 3 lb. was starch, water consumption and also salivation would be much increased. This would cause the starch and the more soluble matter in the corn silage to be washed from the rumen much more rapidly than in the absence of urea. The proportion, in the dry matter of the rumen ingesta, of fibre and of the less soluble protein enmeshed in it would thus be increased.

These experiments of Wegner *et al.* [1941] therefore confirm the views held by the present authors that *in vivo* experiments of this type cannot be expected to yield any certain evidence of protein synthesis from urea until truly representative samples of the total ingesta can be obtained and analysed, and until more is known of the effect of urea on the passage of the various dietary constituents through the rumen.

SUMMARY

1. The relative merits of trichloroacetic acid, sodium tungstate with H_2SO_4, and alcohol have been compared as precipitants in the estimation of N.P.N. in rumen ingesta.

2. A description is given of the methods finally adopted for the estimation of N.P.N., urea and NH_3.

3. The heterogeneous nature of the rumen contents is described and illustrated by analyses.

4. The difficulties involved in the interpretation of the results obtained by *in vivo* experiments are discussed.

5. Owing to these difficulties, the results of *in vivo* experiments of this type cannot be regarded as supplying evidence either for or against the theory that urea is converted to protein in the rumen.

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The Utilization of Urea in the Bovine Rumen. 2. The Conversion of Urea to Ammonia

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(*Received 14 October 1942*)

In the previous paper of this series [Pearson & Smith, 1943] evidence was advanced to show that the interpretation of experiments carried out *in vivo* on the utilization of N.P.N. by ruminants was so difficult as to render the work of little value and that more reliable results could probably be obtained by *in vitro* methods. A plan of investigation was therefore prepared in which samples of rumen ingesta were to be incubated in the laboratory with and without urea, and the N partition studied at intervals throughout the incubation period. At the outset of the work it appeared that even when 40 g. urea were fed per day to a steer with a rumen fistula, no urea could be detected in the rumen ingesta. In preliminary incubation experiments it was also found that 100 g. rumen contents were able to convert some 100 mg. urea to NH_3 in 1 hr. It is probable therefore that any utilization of urea by ruminants involves the conversion of urea to NH_3 as the first stage of the process. A detailed study of this conversion has therefore been made, with particular reference to the effect upon it of various factors such as temperature, *p*H, concentration of urea, nature of the gases present during incubation and certain inhibitory substances. The present paper consists of a brief description of these experiments.

METHODS

Samples of rumen contents were obtained as follows. 2–3 l. semi-solid rumen contents were transferred with an aluminium cup by way of the fistula to a large flask contained in a bucket of sawdust to minimize heat losses. The sample was strained through muslin and the residue pressed by hand. The 'filtrate' so obtained was rich in Protozoa and bacteria and was very similar to the rumen contents as a whole except that the small amount of solid vegetable matter which it contained was in a more finely divided form. The greater part of the solid ingesta of the rumen and reticulum is impregnated with this liquid which, in converting urea to NH_3 and NH_3 to protein, probably behaves very similarly to the more solid ingesta. Since no sampling difficulties arise with this liquid it was used throughout the present experiments and is referred to as the 'rumen liquor'.

Incubation of the rumen liquor was carried out in a water-bath thermostatically controlled at 39°, the body temperature of the bovine. Throughout the greater part of the experimental period the diet of the steer from which the samples were obtained consisted of 14 lb. hay, $2\frac{3}{4}$ lb. oats, 2 lb. bran, 1 lb. starch, 0·7 lb. molasses and 40 g. urea per day with water *ad lib.* The methods of analysis have already been described [Pearson & Smith, 1943].

RESULTS

Influence of CO_2 and N_2 on incubation. One of the objects of the present experiments was to imitate as far as possible in the laboratory the conditions in the intact rumen. In the rumen there are large amounts of carbohydrates such as cellulose, starch, and their breakdown products, and the gas formed in the decomposition of some of these substances usually contains about 62 % CO_2. A few experiments were therefore made to discover whether the nature of the gas and the presence or absence of starch affected the urea-splitting power of the rumen contents. Three 650 ml. aliquots from the same sample were incubated with 100 ml. of a solution containing 1 g. urea, 4 g. K_2HPO_4 and 10 g. glucose. A constant stream of CO_2 was passed through the 1st flask, air through the 2nd and N_2 through the 3rd. Urea was estimated every 10 min. for 2 hr. The results illustrated in Fig. 1 show that the rate of urea conversion was much the same with all three gases. It appeared, however, in the final stages to be slightly more efficient with CO_2 than with air or N_2.

In a 2nd experiment of this type with a much less active rumen liquor, aliquots of the same sample were incubated with air and CO_2, both with and without starch. KH_2PO_4 was present as a buffer but glucose was not included. The results shown in Fig. 2 suggest that CO_2 in the presence of starch accelerates the rate of conversion of urea slightly; otherwise no very significant differences were observed.

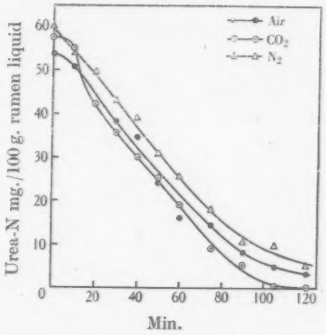

Fig. 1. The conversion of urea in the presence of different gases.

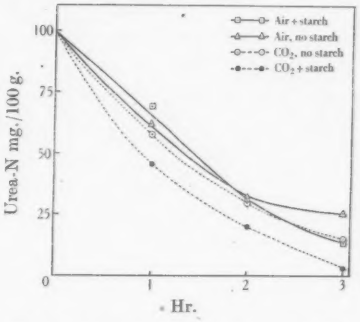

Fig. 2. The effect of CO_2 and starch on the rate of urea conversion.

The effect of starch and phosphate was also tested by incubating them with two different samples of rumen contents in air under strictly comparable conditions. The results are recorded in Table 1.

Table 1. *Ammonia-N produced from urea in 1 hr. at 39°*

(mg./100 g.)

Substances added	Sample 1	Sample 2
Urea alone	27·1	36·7
Starch + urea	31·2	36·5
Starch + urea + phosphate	21·5	30·1
Phosphate + urea	—	30·1

Starch appeared to have a slight accelerating action on the rate of urea conversion with the 1st sample but no effect whatever with the 2nd, which

was the more active of the two. Phosphate obviously had a retarding effect with both samples.

Influence of temperature. 5 ml. of a solution containing 3% KH_2PO_4 and 3% starch were placed in each of ten 200 ml. flasks which were kept in water baths at temperatures ranging from 4 to 89°.

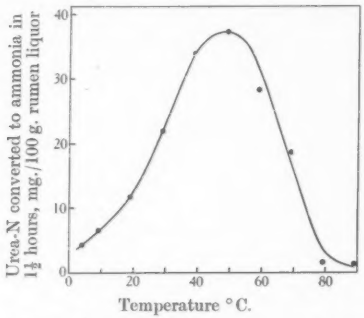

Fig. 3. The effect of temperature on the rate of conversion of urea to ammonia by the rumen liquor.

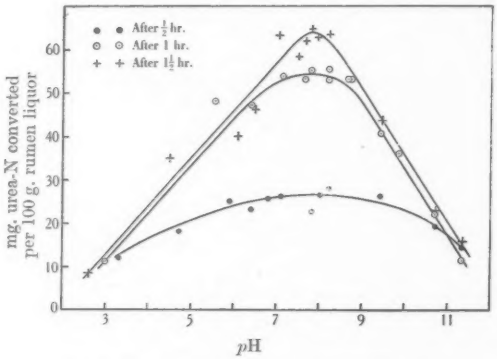

Fig. 4. The effect of *p*H on the urease activity of the rumen contents.

A 50 ml. aliquot from one sample of rumen contents was added to each flask and the contents of the flask were allowed 10 min. in which to reach the temperature of the surrounding bath. Exactly 5 ml. of a solution containing 1 g. urea-N/100 ml. were then added to each flask, and the mixture was incubated for 2 hr. At 10-min. intervals a slow stream of CO_2 was passed through the contents in each flask. At the end of the incubation, urea and NH_3 were estimated. KH_2PO_4 was added in this instance to minimize any loss of NH_3 which might occur at the higher temperatures. The results (Fig. 3) show that urea conversion is insignificant at 4°, but that it increases with increase in temperature and reaches a maximum at 49°. Thereafter it steadily decreases till at 79° there is almost complete inactivity. In two other incubations lasting 1½ and 2 hr. respectively with entirely different samples of rumen liquor, in which no starch was present and through which no CO_2 was passed, the optimum temperature was again 49°.

Influence of p*H.* The technique was similar to that used in the above experiments but the temperature remained constant at 39° while the *p*H in the various flasks ranged from 3 to 11. Three series of incubations were carried out, one for 30 min., another for 1 hr. and a third for 1½ hr. It is probable that during the longer incubations, the salts in the buffer used to obtain a certain *p*H had a more marked effect in retarding urea conversion than the *p*H itself. This may be the cause of anomalous results (see Fig. 4), particularly on the acid side of neutrality. The urease activity of the rumen liquor appears to be very marked over quite a large *p*H range. The optimum probably lies between 7 and 9 and there is little activity below 3 or above 9·5.

The *p*H of the material in the rumen probably varies slightly with a number of factors, but it is rarely below 6·5, and only after a long fast does it approach 7·4. Acid products formed in the rumen are partially neutralized by the incoming saliva, but the buffering capacity of the rumen liquor itself is very high, particularly in view of the fact that its total content of solids is only 2–3%. This is illustrated by the fact that when 5 ml. of approximately *N* HCl were added to 50 ml. rumen liquor the *p*H was 4·7, whereas when 50 ml. of water were used instead of rumen liquor the corresponding value was 1·6.

Influence of urea concentration. In some enzymic reactions the concentration of the substrate has a marked effect on the enzyme activity. To ascertain whether the concentration of urea influenced the urea-converting activity of rumen liquor, various aliquots of the same sample of ingesta were incubated for 1 hr. with different amounts of urea and the liberated NH_3 was estimated. The NH_3 in the tungstate filtrate was not determined by distillation in steam, for with higher concentrations of urea small but significant amounts were converted to NH_3 during the actual

Table 2. *The amount of urea converted to ammonia in varying concentrations of urea*

	(mg. N/100 g.)				
Urea-N in the rumen liquor	30·8	50·0	116	216	414
NH_3-N produced in 1 hr.	17·9	19·2	20·1	20·6	22·2
Urea-N in the rumen liquor	808	1302	1646	3330	
NH_3-N produced in 1 hr.	24·8	25·1	25·4	25·1	

distillation, however accurately the filtrate was neutralized. Prolonged aeration at room temperature in the presence of K_2CO_3 was therefore adopted. The results recorded in Table 2 show that there was a slight increase in the conversion of urea with increased substrate concentration. The effect, however, was not great.

The influence of time of sampling. When urea-feeding is adopted the question arises whether it should be given twice a day with the other concentrates or ingested at more frequent intervals. The following experiments were therefore made to see whether the urea-converting activity of the rumen liquor varied throughout the day.

A sample of rumen liquor removed 1 hr. after a meal was found to convert urea to NH_3 at the rate of 13·4 mg. N/100 g. rumen liquor in 20 min. After a 16 hr. fast the corresponding value was 14·8 mg. There was therefore no significant difference. In a 2nd test a sample taken 17 hr. after the previous meal converted 27·6 mg. urea-N to NH_3 in 1 hr. A meal was then given and samples taken at 2-hourly intervals for 8 hr. During this time the conversion rate remained unchanged at 23·4–23·8 mg. N/100 g. In a 3rd test the value 17 hr. after a meal was 46·3 mg. urea-N/100 g./hr. Samples taken during the next meal and subsequently at hourly intervals gave conversion rates which varied irregularly between 35·4 and 52·3. If the weight of the rumen contents be estimated at 75 kg. these results mean that 40–80 g. of urea could be converted to NH_3 in the rumen in 1 hr. It therefore follows that during urea-feeding, when as much as 100 or 120 g. urea may be ingested in one day, it matters little so far as the urea conversion to NH_3 is concerned whether this be given twice a day in two equal amounts with other concentrates or at more frequent intervals.

Rumen urease

Since the constituents of the diet of the steer during these experiments showed only insignificant amounts of urease activity, and since there is no active enzyme secretion into the rumen except the saliva, the rumen urease must be generated by some of the micro-organisms present in the paunch. From the amounts of urea which can be converted by the rumen liquor in 1 hr. or even in a few minutes at any time throughout the day, it may be assumed that the enzyme is generated in large amounts compared with the rate of growth of the bacteria producing it. Preliminary attempts were made to prepare a sterile but active filtrate of rumen liquor. The filtrate from Berkfeld filters was sterile but without urease activity. With urease extract from jack bean meal also Berkfeld filtration removed the greater part of the urease activity. Since the molecular weight of crystalline urease from soya beans has been found by Sumner, Gralen & Eriksson-Quensel [1938] to be 483,000 it is not surprising that in the present work no filter has been found which will allow the enzyme to pass without the bacteria.

An attempt was made to concentrate the urease activity, and to effect at least a partial separation of the enzyme from the bacteria, by centrifuging. 2 l. of rumen contents were centrifuged for 5 min. at 1000 r.p.m. and 200 ml. of the top portions of the supernatant liquid collected. 200 ml. of the sludge at the bottom of the centrifuge cups, which was extremely rich in Protozoa and bacteria, were also collected. The top layers, the sludge and also some of the original rumen contents which had been centrifuged and then remixed, were each incubated with about 260 mg. urea, and samples were analysed every 30 min. for 2½ hr. The results shown in Fig. 5

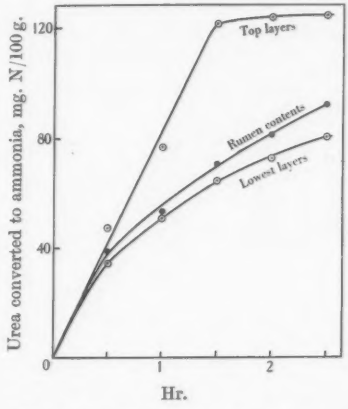

Fig. 5. The rate of urea conversion in the rumen liquor compared with that in the top and bottom layers of the centrifuged rumen liquor.

suggest that for the first ½ hr. or, until some 40 mg. urea-N had been converted to NH_3, the rate of conversion in the lower layers was slightly less than in the rumen liquor, while in the top layers it was slightly greater. At the end of 1 and 1½ hr. the increased rate in the top layers was still more apparent compared with that in the other two. In other experiments in which very much less urea-N was present, and in which conversion was complete in 30–60 min., this difference was by no means clear. The less rapid rate in the rumen liquor and lower layers in the first experiment may have been due less to a smaller content of urease than to a greater concentration of inhibitory substances capable of reducing their urease activity, particularly after the first ½ hr. The results shown in Fig. 5 therefore need not indicate a concentration of urease in the upper layers on centrifuging.

Toluene, which is known sometimes to destroy bacteria but to permit enzyme action, was found to be without effect on the urease activity of the rumen liquor, but here it is not certain whether the urea-converting bacteria were completely destroyed by that treatment. Reference will be made to this in a subsequent paper. 30% alcohol extracts of the urease were inactive.

Urease activity was also inhibited by small quantities of substances such as quinone and NaF. Quastel [1933] has shown that the activity of ordinary urease preparations is readily inhibited by quinone, and that the inhibition can be prevented by thiol compounds such as H_2S and cysteine. Samples of rumen liquor containing urea were therefore incubated for 1 hr. with varying amounts of quinone and with quinone in the presence of H_2S and cysteine (Tables 3 and 4). With one sample of

Table 3. *The inhibitory effect of quinone on rumen urease and its neutralization by H_2S*

Urea-N converted to NH_3 (mg.)

	Sample 1		Sample 2	
Quinone %	Quinone	Quinone + H_2S	Quinone	Quinone + H_2S
0·0	71·1	71·4	61·2	58·6
0·005	26·8	69·3	13·1	50·9
0·0075	14·2	69·7	2·4	49·9
0·01	13·2	68·2	1·0	33·5
0·03	5·0	10·4	—	—
0·05	3·0	4·3	—	—

Table 4. *The influence of cysteine on the urease-inhibiting action of quinone*

0·01% quinone + cysteine (%)	0·0	0·005	0·01	0·03
Urea-N converted to NH_3 in 1 hr. (mg.)	1·6	25·2	64·4	67·5
0·01% quinone + cysteine (%)	0·05	0·07	0·10	
Urea-N converted to NH_3 in 1 hr. (mg.)	64·4	66·1	62·6	

0·05% cysteine in the absence of quinone gave a value of 63·5.

rumen liquor concentrations of quinone as low as 5 in 100,000 substantially reduced the urease activity. At higher concentrations the degree of inhibition increased and ultimately approached completion at 3 parts in 10,000. With sample 1 H_2S neutralized the inhibitory action of quinone up to concentrations of 0·01%. With sample 2 the neutralization of inhibition was marked but not quite complete. Cysteine at concentrations of 0·01% or above also completely neutralized the inhibitory action of 0·01% quinone (Table 4). Cysteine itself was found to have a very slight inhibitory effect. Thus 0·05% cysteine in rumen liquor reduced the rate of urea conversion from 47 to 43 mg. N/100 g. rumen liquor/hr. These results for urease activity of rumen liquor are similar to those of Quastel [1933] for urease from jack bean meal, and show that the enzymes from these two very different sources have much in common and may be identical.

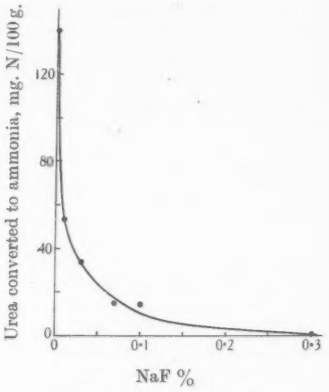

Fig. 6. The inhibition of urease activity in the rumen liquor by sodium fluoride.

Mystkowski [1928] has shown that NaF is a powerful inhibitor of ordinary urease. This was found to be true also for the urease of the rumen. Aliquots of a sample of rumen liquor containing urea were incubated for 2 hr. with various concentrations of NaF. At a concentration of 0·01% the activity was reduced by about 50% and at 0·3% inhibition was complete (Fig. 6). This effect is due entirely to the fluoride radical, for in a control experiment with 0·5% NaCl there was no inhibition. A similar experiment was carried out with boric acid and borax (Table 5). 0·05% borax had

Table 5. *The inhibition of urease activity by boric acid and borax. Urea conversion to NH_3*

(mg. N/100 g.)

Boric acid or borax %	Boric acid		Borax	
	30 min.	60 min.	30 min.	60 min.
None	28·5	46·8	28·5	46·8
0·05	21·7	40·6	28·3	48·2
0·10	19·5	36·5	24·3	43·3
0·50	11·7	22·3	13·0	25·0

With further samples of rumen liquor 1 and 2% boric acid and borax completely inhibited the urease activity.

no inhibitory effect and may even have stimulated the urease action slightly. Above this concentration, however, the amount of urea conversion was dimin-

ished significantly. At the lower concentrations inhibition with boric acid was greater than with borax.

SUMMARY

1. In the utilization of urea by dairy cattle the first stage is probably the conversion of urea to NH_3 in the rumen. Moreover, the urease activity of the rumen ingesta is so great at all times of the day, whatever the time of feeding, and remains so little affected by relatively large amounts of urea, that all the urea ever likely to be fed, even to a high yielding cow in full lactation, would readily be converted to NH_3 within 1 hr. This fact has been confirmed by Wegner, Booth, Bohstedt & Hart [1941], who found a marked increase in N.P.N. and NH_3 in the rumen after a urea meal but were unable to detect urea itself.

2. The urease of the rumen resembles, in activity, that from soya and jack beans. Changes in its activity with changes in temperature and *p*H, and its behaviour in the presence of such inhibitors as quinone and NaF, are typical of enzymes of the urease type. Preliminary attempts to obtain enzyme preparations free from bacteria have, however, proved unsuccessful.

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The Utilization of Urea in the Bovine Rumen. 3. The Synthesis and Breakdown of Protein in Rumen Ingesta

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(*Received 14 October 1942*)

The urease activity of bovine rumen ingesta has been studied in detail by Pearson & Smith [1943*b*] who showed that the urease activity of the rumen is sufficient to convert rapidly to NH_3 all the urea ever likely to be included in the diet as a partial substitute for protein. The utilization of urea by the ruminant probably takes place in two stages, first the conversion of urea to NH_3 and second the conversion of NH_3 to protein. The present section of the work was undertaken to investigate this 2nd stage. Incubations of rumen contents were to be carried out *in vitro* under a variety of different conditions with and without urea, to obtain as much evidence as possible by chemical analysis either for or against the theory of protein synthesis in the rumen from non-protein N. Samples were to be taken for direct microbiological examination by Mr Frank Baker [Baker, 1942 *a*, *b*; 1943] and for plate counts by Dr C. Higginbottom to discover whether the protein synthesis, if it occurred, were accompanied by an increase in the numbers of micro-organisms present.

First, it had to be established by chemical means whether any protein synthesis could be detected, and if so, to what extent it was affected by the presence of ordinary dietary constituents such as carbohydrates, proteins and amino-acids, as well as by substances which might be of a toxic nature. The present paper is confined to a description of this particular aspect of the work.

METHODS

The method of obtaining samples of rumen liquor and the incubation and analytical procedures have already been described [Pearson & Smith, 1943 *a*, *b*].

Long-period incubations. It seemed possible that if any measurable change in N partition took place on incubating rumen contents *in vitro*, the changes would be more readily detected in fairly long incubations. Hence preliminary incubations with rumen contents were carried out, each lasting several days (Fig. 1). 900 ml. rumen liquor were incubated with 2·5 g. urea, 12 g. K_2HPO_4, 10 g. glucose and 0·1 g. $FeSO_4$ for 8 days. Note that while the total N remained constant throughout, the N.P.N. varied considerably. For the first 3–4 days it changed very slightly, but between the 3rd and 7th days it greatly decreased. Assuming that the difference between total N and N.P.N. is protein N, an assumption which is discussed later, Fig. 1 shows that the amount of protein in the medium changed little at first, but that during the last 5 days it increased

substantially. The explanation may be that many micro-organisms in the rumen ingesta ceased to multiply on removal from the rumen and incubation, but that after 3–4 days the strains which had survived and for which the stagnant rumen contents were a suitable medium passed from a stationary phase to a phase of active growth in which rapid multiplication occurred. Several experiments of this type were performed with and without urea, and although the results varied in detail, the general trend of the curves obtained was always the same.

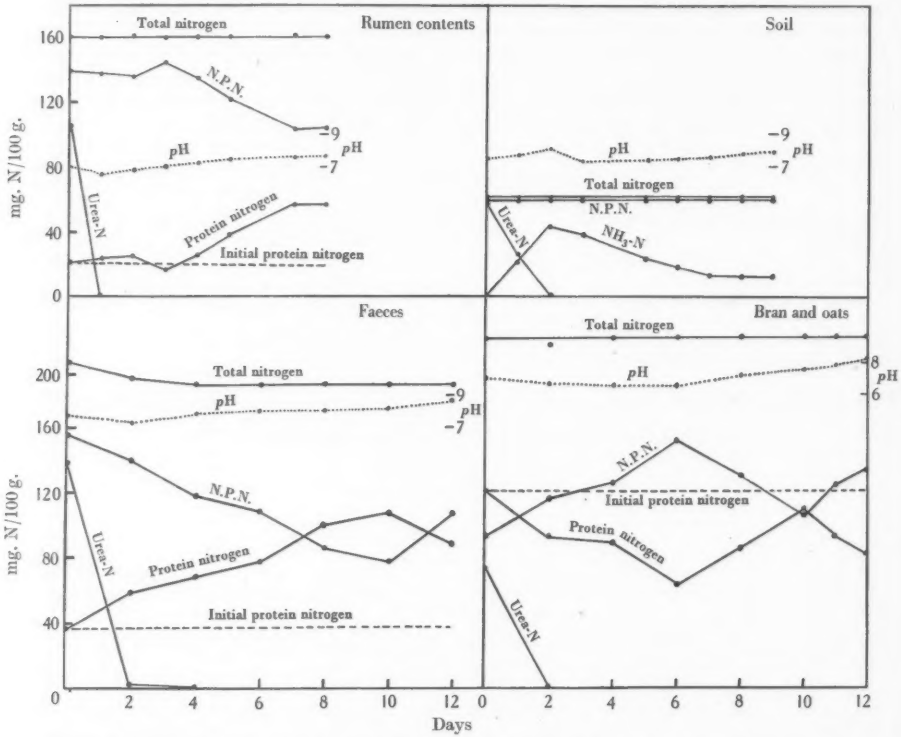

Fig. 1. The results obtained from prolonged incubations of rumen contents and also from prolonged incubations of suspensions of faeces, feeding-stuffs and soil. The amount of protein synthesis with rumen contents and faeces can be found by comparing the initial protein nitrogen with the protein nitrogen observed at different times throughout the incubation period. (In order to make the diagrams as simple as possible NH_3-N is not shown for rumen contents, feeding-stuffs and faeces. In all three instances after urea was converted to ammonia, the NH_3-N followed a course parallel to that of the N.P.N.)

To ascertain whether these changes in N.P.N. were associated with the presence of micro-organisms in the rumen contents or were fortuitous changes inherent in the experimental procedure, Mr Frank Baker suggested that parallel experiments be done with bovine faeces, with soil suspensions and also with suspensions of bran and oats in which the bacterial content would be widely different from that of rumen liquor. Bran and oats were chosen because they formed an appreciable part of the diet of the steer used. 100 g. faeces, 100 g. soil and finally 50 g. bran mixed with 50 g. oats were each suspended in 1 l. water containing urea, phosphate, glucose and $FeSO_4$ in amounts similar to those added to the rumen contents (Fig. 1). With the faeces the apparent increase in protein was much greater than with the rumen contents, and the increase was rapid from the beginning of the incubation, whereas with the rumen contents there was an initial lag of 2–3 days. The explanation may be that the bacteria present in the faeces in such large numbers consist of types for which the stagnant faecal suspension is an ideal medium and that the faeces contain few, if any, of those types present in the rumen which die so readily when removed thence and which are very difficult to grow on the usual types of media. It is therefore not surprising that in these prolonged incubations there is an initial lag period with the rumen liquor but not with the faeces.

The soil suspensions were by no means sterile, for they brought about a marked conversion of urea to NH_3 and of NH_3 to nitrate (Fig. 1). No change in N.P.N. could, however,

be detected. The reason is probably that the number of bacteria found in soil suspensions of this kind is so small compared with the numbers present in the faeces and rumen contents that they could multiply very greatly and utilize N.P.N. without causing any measurable decrease in the N.P.N. of the medium. These three types of experiment suggest therefore that the chemical changes denoting increase in protein when faeces and rumen ingesta are incubated may be associated with the micro-organisms they each contain.

With bran and oats the results were more complex. Fermentation set in early and was accompanied by much frothing and a very foul smell. During the first 6 days fermentation resulted in a considerable decrease in the protein content (Fig. 1). After that, however, the protein increased again but never attained the original value. This increase at the later stages was possibly due to some strains of bacteria passing from a stationary phase to a phase of more active growth.

Short-period incubations. Samples from the long-period incubations described above were examined microbiologically by Baker [1942*a*, *b*; 1943] who stated that even after 1 day's incubation great changes had taken place in the microflora and fauna, so that the 'microbiological picture' at the end of 1 day bore little resemblance to that in the initial sample. Experiments were therefore carried out to determine how long samples of rumen contents could be incubated without detectable microbiological change. Samples of rumen ingesta from these experiments were examined by Mr Baker who found no significant change during the first 2–4 hr. of incubation. In all subsequent work therefore incubation periods of 2–4 hr. were adopted, since it was essential, if the work were to have any practical bearing, that any protein synthesis detected should occur under conditions as similar as possible to those in the intact rumen (Fig. 2).

Approximately 1500 ml. rumen liquor were incubated with 0·65 g. urea, 4·5 g. K_2HPO_4, 0·1 g. $FeSO_4$ and 7·5 g. starch. The *p*H was controlled when necessary by the addition of a few drops of concentrated Na_2CO_3 solution. From Fig. 2 the following facts appear. (1) The total N remained unchanged. (2) Urea was rapidly converted to NH_3. (3) Relatively little N.P.N. was present except as urea or NH_3. (4) During the first 3 hr. when the microbiological conditions in the medium appeared to resemble closely those of the rumen, synthesis of protein appeared to occur to the extent of about 9 mg. N/100 g. rumen liquor. (5) There was a decrease in N.P.N. accompanied by a parallel decrease in ammonia N, which suggests that the synthesized protein was produced from the ammonia N and not from the N.P.N. present in other forms.

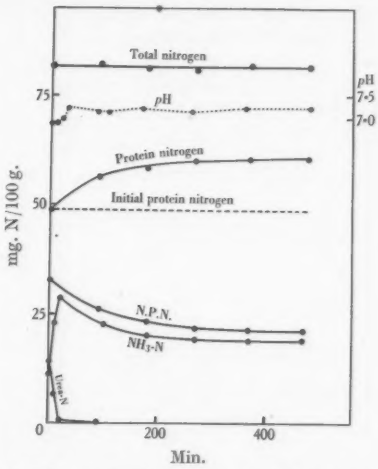

Fig. 2. The results obtained in a typical short period incubation of rumen liquor.

Table 1. *Comparison between sodium tungstate and trichloroacetic acid methods of estimating* N.P.N.

mg. *N*/100 g. rumen contents

Incubation no.	Initial N.P.N.			Final N.P.N.			Synthesis	
	Tungstate	Trichloroacetic acid	Difference	Tungstate	Trichloroacetic acid	Difference	Tungstate	Trichloroacetic acid
1	120·1	128·7	8·6	111·3	119·8	8·5	8·8	8·9
2	112·0	119·4	7·4	107·8	116·4	8·6	4·2	3·0
3	115·0	124·6	9·6	111·7	120·3	8·6	3·3	4·3
4	113·4	119·8	6·4	106·6	115·2	8·6	6·8	4·6

Table 2. *Protein synthesis estimated by the tungstate and trichloroacetic acid methods*

Total N = 143·5 mg./100 g. rumen liquor

Time min.	Tungstate method		Trichloroacetic acid	
	N.P.N. (initial)	Synthesis	N.P.N. (initial)	Synthesis
	(mg. N/100 g. rumen liquor)			
Initial	94·9	—	107·8	—
20	93·7	1·2	105·9	1·9
80	88·8	6·1	103·5	4·3
90	88·1	6·8	100·6	6·2
100	87·6	7·3	99·7	8·1
180	87·4	7·5	98·0	9·8
290	86·9	8·0	99·6	8·2

This amount of protein synthesis appears at first sight to be very small. If, however, the contents of the rumen be estimated conservatively at 75 kg., this value of 9 mg. N/100 g. rumen contents in 3 hr. is equivalent to the conversion of 54 g. N to 337 g. protein in 1 day. 54 g. N would be equivalent to some 25% of the total protein requirements of a cow yielding 2–3 gallons of milk per day.

Control experiments were carried out with bran and oats, faeces and soil but no change in N.P.N. was ever found during the first 3 hr. of incubation. rumen liquor with certain toxic substances such as NaF. In a typical experiment 2·4 l. rumen liquor were divided into 4 equal parts. One part was used as a control while to the other 3 parts NaF was so added that one contained 0·01%, another 0·1% and a third 1%. To all 4 parts, 100 ml. solution containing 1 g. urea and 3 g. starch were added. These mixtures were then incubated for 4 hr. with a slow stream of CO_2 passing through them. Samples were removed for analysis initially and at the end of each hour (Fig. 3, A). In two other similar

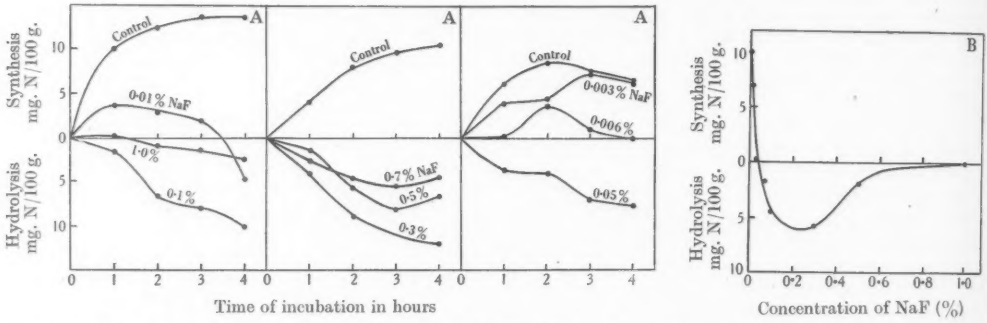

Fig. 3. A, the amount of synthesis or hydrolysis observed with different concentrations of NaF compared with controls incubated in the absence of NaF (first NaF experiment). B, the amount of synthesis or hydrolysis observed after incubation for 4 hr. with different concentrations of NaF (second NaF experiment).

The apparent synthesis observed with rumen contents seems therefore to be peculiar to the rumen ingesta.

Synthesis of some protein has always been observed in normal incubations of this type, both with tungstate and the CCl_3.COOH procedure outlined in a previous paper [Pearson & Smith, 1943*a*]. From Tables 1 and 2 it will be observed that CCl_3.COOH gave values for N.P.N. which were 6–13 mg. higher than those obtained by acid sodium tungstate, but nevertheless the amount of protein synthesis estimated by the two methods was of the same order.

When the occurrence of a decrease in N.P.N. in such incubations had been repeatedly confirmed, the question arose whether this was due to protein synthesis or to some other cause. If synthesis were the explanation, questions arose as to whether it was indeed due to micro-organisms, and whether its magnitude was affected by the presence of carbohydrates, proteins and amino-acids.

The microbiological nature of the synthesis

Proof that the changes in N partition observed on incubation were biological in origin was first obtained.

Influence of sodium fluoride. The first method of approach was to study the effect of incubating experiments the concentrations of NaF were 0·003, 0·006, 0·05, 0·3, 0·5 and 0·7%. The data in Fig. 3 show that protein synthesis was strongly inhibited by as little as 0·01% NaF, and that with a concentration of 0·1%, protein synthesis was replaced by hydrolysis. With 1·0% hydrolysis was almost inhibited in the first instance and completely inhibited in the second.

When two 500 ml. portions of the same rumen liquor were incubated for 3 hr. with 1·8 g. starch and 0·3 g. urea, one containing 0·5% NaCl, the other 0·5% NaF, the results showed that 0·5% NaCl had no effect on the synthesis occurring in the 3 hr., whereas with 0·5% NaF (Fig. 3) hydrolysis

Table 3. *Protein synthesis in presence of NaCl*

	Control mg. N/100 g.		0·5% NaCl mg. N/100 g.	
	Total N	N.P.N.	Total N	N.P.N.
Initially	103·5	33·0	103·7	33·4
After 3 hr.	103·3	27·3	103·4	27·4
Synthesis	—	5·7	—	6·0

predominated over synthesis. The action of the NaF was therefore a specific one associated with its recognized inhibitory action on enzymes and micro-organisms.

It appears therefore that the change during incubation in the difference between total N and N.P.N. is the resultant of two different processes, one synthetic, the other hydrolytic, and that both are of microbiological origin. As the concentration of NaF increases, synthesis diminishes and becomes completely masked by hydrolysis. When, however, the concentration of NaF increases above 0·2 or 0·3 % hydrolysis also wanes; ultimately, at a concentration of 1–2 % NaF, the rumen liquor becomes 'fixed'.

Table 4. *The effect of quinone on protein synthesis*

Quinone %	Synthesis (+) or hydrolysis (−) mg. N/100 g.
None	+12·2
0·001	+ 8·5
0·005	+ 5·4
0·01	+ 5·1
0·05	− 6·4
0·01 + H_2S	+10·5
H_2S + water alone	+10·9

Table 5. *The effect of boric acid and borax on protein synthesis*

	Synthesis mg. N/100 g. rumen liquor			
Conc. of boric acid or borax %	Sample 1		Sample 2	Sample 3
	Boric acid	Borax	Boric acid	Borax
Control	9·1	9·1	5·9	14·0
0·01	—	—	5·8	10·2
0·03	—	—	3·0	—
0·05	3·6	2·2	—	—
0·06	—	—	4·2	—
0·10	1·4	1·6	3·2	4·7
0·30	—	—	−0·2*	0·9
0·50	0·5	0·2	—	—
1·00	—	—	0·0	−3·0*
2·00	—	—	—	−4·4*

* Hydrolysis.

Influence of quinone. A similar experiment was made with quinone alone and with quinone in the presence of H_2S (Table 4). Synthesis was markedly diminished in the presence of even 0·005 % quinone, and at a concentration of 0·05 % hydrolysis predominated. In the presence of H_2S the quinone appeared to lose its toxic properties. This agrees with the behaviour of quinone in the presence of thiol compounds previously recorded for soya bean urease by Quastel [1933], and also observed with rumen-urease activity by Pearson & Smith [1943*b*].

Influence of boric acid. Two experiments similar to those with NaF and quinone were performed, one with boric acid and the other with borax. Table 5 shows that synthesis was again greatly diminished and that with the two highest concentrations of borax hydrolysis predominated.

Influence of temperature. All the above experiments suggested that the observed protein synthesis was the result of biological processes. If so, changes in temperature might also be expected to affect the magnitude of the synthesis. Different portions of the same sample of rumen liquor were therefore incubated with urea and starch for 1½ hr. at temperatures varying between 0 and 90°. Fig. 4 shows

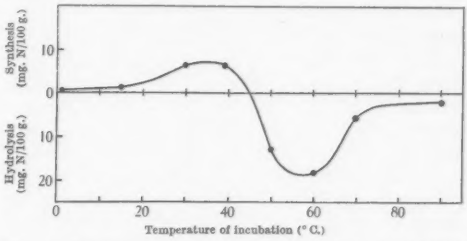

Fig. 4. The amount of synthesis or hydrolysis obtained by incubating rumen liquor for 1½ hours at different temperatures.

that at temperatures below 16° little change occurred, but that at 30 and 40° the usual amount of protein synthesis took place. When, however, the incubation temperature was 50° hydrolysis predominated. Hydrolysis attained a maximum at about 60° and was almost entirely inhibited at 90°. From 40 to 90° the curve is very similar indeed to that obtained with increasing concentrations of NaF (Fig. 3), except that hydrolysis in the absence of NaF attained a greater magnitude at 60° than was observed with any concentration of NaF at 39°. This difference may be due to the use of different samples of rumen contents in the two instances. However, the higher temperatures (up to 50 or 60°) may greatly inhibit synthesis but may actually increase the hydrolytic processes, whereas NaF tends to inhibit both, the more sensitive synthetic process disappearing first.

Influence of toluene. Pearson & Smith [1943*b*] have shown that toluene has no effect on the urease activity of the rumen liquor. A test was therefore carried out to see whether synthesis is affected by this substance. Two 500 ml. portions of the same sample of rumen liquor were incubated with starch and urea for 3 hr. To one sample were added 50 ml. toluene. The control gave a synthesis of 7·4 mg. N/100 g. rumen liquor, whereas with toluene hydrolysis occurred amounting to 13·7 mg. N/100 g. This again shows how readily synthesis can be diminished, allowing hydrolysis to predominate. The urease activity in this instance remained unaffected.

Effect of filtration. Rumen liquor was filtered through a Chamberland filter type 'F'. The clear filtrate was then incubated in the presence of 0·4 % starch and approximately 0·5% NH_4HCO_3. Since

Pearson & Smith [1943*b*] had already shown that a filtrate of this type possessed no urease activity, the N was added in the form of NH_4HCO_3 and not as urea. In the absence of urease activity noted in previous work it was not surprising that neither synthesis nor hydrolysis was detected in this experiment, which again suggests that the synthetic and hydrolytic processes consistently observed in these incubations are to be associated with the micro-organisms of the rumen.

The effect of fractionation by centrifuge. On allowing the rumen liquor to stand for even a very short time, the Protozoa and the larger bacteria tend to sediment. On centrifuging for 5 min. at 1000 r.p.m. this tendency is very marked indeed. The bottom layer consists of a sludge rich in Protozoa and large bacteria which stain blue with iodine [Baker, 1942*a*, *b*; 1943]. An experiment was therefore carried out to see whether the amount of synthesis was greater or less in the top and bottom fractions of the rumen liquor than in the sample of rumen liquor as a whole. At the same time the effect of keeping the rumen liquor and its fractions at approximately 0° for 24 hr. before incubation, was studied. A 3 l. sample of rumen liquor was centrifuged in 250 ml. cups for 5 min. at 1000 r.p.m. The contents of two of the cups were then thoroughly remixed by shaking. One portion of the remixed ingesta was incubated with 1 g. starch and 0·5 g. urea as the control. The other was first kept in the refrigerator for 24 hr. and then treated in the same way. 50 ml. of the liquor at the top and 50 ml. of the sludge at the bottom were then removed from each of the ten remaining centrifuge cups. The top fractions were mixed together and half of the sample incubated immediately with starch and urea, the other half being placed in the refrigerator for 24 hr. before incubation. The bottom fractions were similarly treated. From the results in Table 6 two main facts emerge. First, that

Table 6. *Protein synthesis with two fractions of rumen liquor separated by centrifuging*

	Synthesis (+) or hydrolysis (−) mg. N/100 g.	
	Immediately	After 24 hr. at 0°
Rumen liquor	+11·1	+2·8
Top fraction	+14·7	+5·0
Bottom fraction	+ 7·2	−2·5
Arithmetical mean of top and bottom fractions	+10·9	+1·3

holding the rumen contents at 0° for 24 hr. greatly reduces the amount of synthesis not only in the total rumen liquor but also in the top and bottom fractions—a fact which again suggests that this synthesis is associated with certain of the micro-organisms present in the rumen which are readily inactivated. Secondly, that on dividing the rumen liquor into 2 fractions by centrifuging, the top portions show very much greater amounts of synthesis than the bottom portions, whether the various liquors are incubated immediately or after 24 hr. at 0°. The possible significance of this finding is discussed later.

From all these experiments it appears that when rumen liquor is incubated with starch and urea at 39° the N metabolism of the system involves both synthetic and hydrolytic processes which probably proceed simultaneously. These processes are microbiological in origin and the equilibrium between them is very readily changed, so that either synthesis or hydrolysis predominates according to the conditions during the incubation. This last point is of great importance in connexion with urea utilization, for if the processes in the rumen at any time are predominantly hydrolytic with NH_3 as the end-product, the utilization, if any, must be due to processes other than the conversion of these substances to protein in the paunch. Factors which might be expected to influence the magnitude of the synthesis and its predominance over hydrolysis were therefore investigated next.

Influence of p*H.* The *p*H of the rumen ingesta varies slightly from time to time but it is rarely below 6·3 or above 7·4. In many of the incubations carried out during this work *p*H determinations have been made, and the values have usually been between 6·3 and 7·4, even after incubation for 3–4 hr. at 39°. No correlation was found between the *p*H during incubation and the amount of protein synthesis, provided the *p*H lies roughly between the above limits. Thus three samples of the same rumen liquor were incubated for 5½ hr. with 4 g. K_2HPO_4, 0·1 g. $FeSO_4$, 5 g. glucose, 5 g. starch and approximately 1 g. urea/l., CO_2 being passed through each flask throughout the incubation period. In the case of the 1st sample (M) the *p*H was measured at frequent intervals and adjusted by adding the correct amount of a saturated solution of Na_2CO_3. To the 2nd sample (N) the same amount of Na_2CO_3 was added at the same times as to M, without previously removing samples to measure the *p*H. To the 3rd sample (P) 10 g. powdered $CaCO_3$/l. were added initially. Fig. 5 shows that the *p*H in the first two samples was fairly close to the figure of 7·2 throughout, whereas in the 3rd sample the initial value of 7·2 decreased during the incubation to 6·3. Solid $CaCO_3$ is therefore of little value in maintaining the initial *p*H. The amount of synthesis in M could not be measured accurately since so many samples were removed to measure the *p*H before and after adjustment, but the results for N and P show that the amount of synthesis when the *p*H remained constant did not

differ significantly from the amount observed when the *p*H fell from 7·2 to 6·3.

In the previous papers of this series the great buffering power of the rumen liquor is discussed. In view of this it seemed unnecessary to study the effect on synthesis of hydrogen-ion concentrations outside the normal range of 7·4 to 6·3, particularly as it was found that synthesis may be affected as much by the type of buffer employed to obtain the *p*H as by the *p*H itself. That synthesis can be markedly affected, however, by an abnormally high *p*H is shown in the experiments which follow, in which incubations were carried out in the presence of different gases.

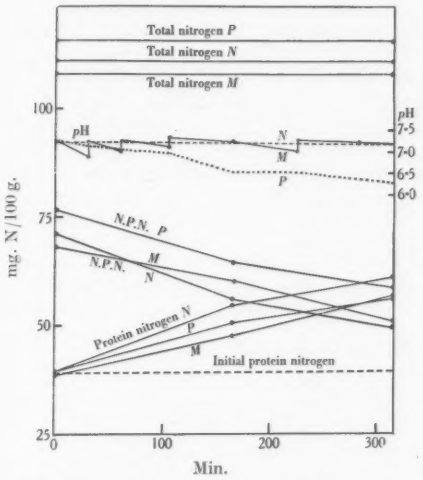

Fig. 5. The amount of synthesis obtained when the *p*H is kept constant at 7·2 compared with that obtained when the *p*H is allowed to decrease from 7·2 to 6·3.

Influence of CO_2-N_2. The composition of the gas in the rumen varies slightly from time to time, but typical analytical figures such as those found by Olson [1940] are: CO_2 62%, CH_4 15%, N_2 18% and O_2 1%. In view of the great preponderance of CO_2 and the low concentration of O_2 in these gases, experiments were carried out to find whether the nature of the gas passing through the medium had any influence on the amount of synthesis. One sample of rumen liquor was divided into 6 portions. Three of these were incubated for 3 hr. with 3 g. of starch and 800 mg. urea-N/l. while the other 3 were incubated with starch only. There were thus 3 pairs of samples, one in each pair containing added urea and the other containing no urea. A slow stream of CO_2 was passed through the 1st pair, N_2 through the 2nd and air through the 3rd. From the results in Table 7 the following points emerge. (1) Without added urea, protein synthesis in the presence of CO_2 was so little greater than that with N_2 and air that it is doubtful if it was significant. (2) When approximately 800 mg. urea-N/l. were present, protein synthesis in the presence of CO_2 was substantial, but with N_2 and air hydrolysis predominated. (3) Hydrolysis occurred with N_2 and air perhaps because with these two gases the *p*H increased during the incubations to 8·7, whereas with CO_2 it remained between 6·8 and 6·6. To see whether this predominance of hydrolysis was due to the nature of the gases or to the change in *p*H, a second experiment was carried out in which the amount of urea-N added to 3 of the 6 flasks was only 150 mg./l. In this instance the *p*H of the N_2 and air samples did not exceed the initial value of 6·9. From Exp. 2 (Table 7) the following conclusions may be drawn. (1) In the absence of added N.P.N. synthesis in air and in N_2 was not significantly less than that obtained with CO_2. (2) With an amount of urea insufficient to cause an appreciable change in *p*H when the urea was converted to NH_3, CO_2 and N_2 gave very similar amounts of synthesis. But with air there was significantly less. It is clear from these two experiments that the hydrolysis in air and in N_2 in Exp. 1 was due to the rise of *p*H and not primarily to the gases themselves. From many similar experiments the authors concluded

Table 7. *Effect of different gases on protein synthesis*

		Exp. 1		Exp. 2	
		*p*H	Synthesis (+) or hydrolysis (−) mg. N/100 g.	*p*H	Synthesis (+) mg. N/100 g.
Without added urea	CO_2	6·8–5·9	+ 3·9	6·9–5·9	+4·9
	N_2	6·8–6·3	+ 2·1	6·9–6·3	+4·6
	Air	6·8–6·6	+ 2·1	6·9–6·2	+4·7
80 mg. urea N/100 g. rumen liquor in Exp. 1 and 15 mg. in Exp. 2	CO_2	6·8–6·6	+14·0	6·9–6·1	+7·9
	N_2	6·8–8·7*	− 9·2	6·9–6·6	+8·2
	Air	6·8–8·7*	− 4·2	6·9–6·8	+5·4
No urea; 0·1 g. K_2HPO_4/100 g.	CO_2	—	—	7·1–6·1	+5·0

* At *p*H 8·7 NH_3 escapes from the medium during the incubation. In estimating the amount of hydrolysis the loss in total nitrogen is therefore allowed for.

that the greatest synthesis was always obtained when CO_2 was passed slowly through the medium, CO_2 having the advantage over N_2 that in the presence of reasonably small amounts of urea or NH_4HCO_3 it prevented an unfavourable increase in *p*H.

In Exp. 2 (Table 7) the *p*H of the sample incubated with CO_2 with no added urea decreased from 6·9 to 5·9. Hence protein synthesis might have been significantly greater than 4·9 mg. N/100 g. had the *p*H not diminished so far. A 7th incubation was therefore carried out simultaneously in which 0·1 g. K_2HPO_4/100 g. rumen contents was present. The *p*H in this instance diminished only to 6·1 but protein synthesis remained unchanged.

Influence of dietary factors on the utilization of non-protein nitrogen

NH_4HCO_3 and $(NH_4)_2SO_4$ as urea substitutes. Because urea is now indispensable to many industries it is less readily available as an animal feeding-stuff. NH_4HCO_3 and $(NH_4)_2SO_4$ are more plentiful and more readily prepared on a large scale. Incubations were therefore carried out to see whether with these substances protein synthesis equalled that obtained with urea. 3 g. starch were present per l. rumen liquor in every instance, and a slow stream of CO_2 was passed through all the flasks. Incubation lasted 2 hr. Two series of experiments were made. In one the amount of N.P.N. added in the different forms was 90–100 mg./100 g., in another it was about 15 mg./100 g. It can be seen from the results (Table 8), first, that the amount of synthesis with these particular samples of rumen liquor was increased by the addition of N.P.N. and secondly, with NH_4HCO_3 it was equal to that with urea and greater than that with $(NH_4)_2SO_4$. It is not clear why sulphate should be less efficient than bicarbonate in promoting protein synthesis. This difference was, however, only significant in the first instance when a relatively large amount of sulphate was added, but in practice the quantity of $(NH_4)_2SO_4$ fed to a lactating cow to replace a portion of the dietary protein would be equivalent, if given in two equal portions per day, to only about 25 mg. N/100 g. of rumen contents, even immediately after feeding. It is doubtful, therefore, if there would be any detectable difference in the efficiency of the utilization of these three different forms of N. On the basis of the present results, however, preference should be given to urea or NH_4HCO_3.

Influence of urea concentration. In order to see whether urea concentration had much effect on the amount of protein synthesis a series of incubations was carried out in which 1 large sample of rumen liquor was divided into 7 equal portions and different amounts of urea added to each. Incubation lasted 3 hr. in a slow current of CO_2; 3 g. starch were added per l. rumen liquor. Table 9 shows that under these conditions the maximum amount of synthesis is obtained when the added urea-N amounts to 75 or 100 mg./100 g. Below and above these concentrations the amount of synthesis was significantly less. Since urea is so rapidly converted to NH_3 in the rumen liquor, these results should be equally true for NH_4HCO_3.

Table 8. *Protein synthesis with urea, NH_4HCO_3 and $(NH_4)_2SO_4$**

	Exp. 1			Exp. 2		
Supplement	N.P.N. added (mg. N/100 g.)	Protein synthesized (mg. N/100 g.)	*p*H	N.P.N. added (mg. N/100 g.)	Protein synthesized (mg. N/100 g.)	*p*H
—	—†	4·0	6·7–5·7	—†	1·3	6·5–5·6
Urea	100	11·5	6·7–6·8	16	5·1	6·5–5·9
NH_4HCO_3	80	11·3	7·2–6·7	15	5·4	6·5–5·6
$(NH_4)_2SO_4$	85	6·3	6·5–5·8	15	4·5	6·5–5·9

* Analar. † N.P.N. of rumen contents with no addition was initially 11·4 in Exp. 1 and 6·9 in Exp. 2.

It early became clear that the utilization of N.P.N. by the ruminant may depend on the nature of the diet as a whole, and particularly on whether the entire ration is correctly balanced. If adding urea upsets the proper proportions of protein and potential protein to carbohydrate, the urea itself may not be efficiently utilized. This may explain some of the conflicting results cited in Krebs's [1937] review and which have been obtained from time to time during the past 40 years in experiments in which N.P.N. has been added to the diet of rumi-

Table 9. *Influence of concentration of urea on protein synthesis*

Urea added (mg. N/100g.)	Synthesis mg. N/100 g.	Final *p*H*
—	5·2	5·7
50	7·8	6·2
75	11·3	6·4
100	11·2	6·6
150	6·4	7·1
400	6·7	7·6
650	2·0	7·8

* The initial *p*H in this instance was 6·2 throughout.

nants. Presumably the greatest efficiency of urea as a partial protein substitute would be shown when the dietary protein equivalent was low compared with the starch equivalent. In such a diet starch itself would probably be present in relatively large amounts. The effect of different concentrations of starch and also of different carbohydrates on the amount of protein synthesis observed in the *in vitro* incubations was therefore studied. The results were of such interest that a further series of experiments was made in which the effect of different proteins and amino-acids was studied.

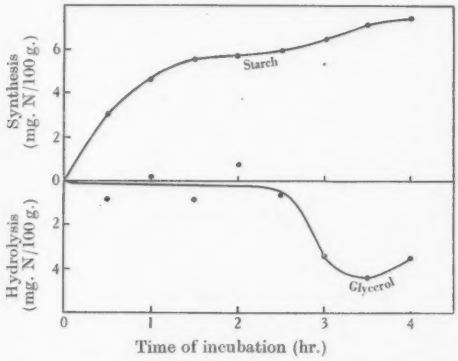

Fig. 6. The synthesis obtained by incubating rumen liquor in the presence of 0·3% starch compared with the hydrolysis obtained when starch is replaced by glycerol of the same concentration.

Influence of concentration and nature of carbohydrates. Preliminary tests showed that when 3 g. starch/l. were added to the rumen liquor the amount of synthesis was always greater than when rumen liquor was incubated alone. Moreover, when starch was replaced by glycerol synthesis was very restricted in amount and was sometimes replaced by hydrolysis. Typical results for an experiment in which two samples of the same rumen liquor were incubated, one with starch and one with glycerol (both 3·6 g./l.) are shown in Fig. 6. 2·4 g./l. urea were present and CO_2 was passed through the samples during incubation. Whereas with starch protein synthesis was equivalent to 7·4 mg. N/100 g., glycerol was responsible for hydrolysis amounting to 3·5 mg. N/100 g. Four separate portions of the same rumen liquor were therefore incubated at one time with different carbohydrates (Exp. 1, Table 10). One contained 3 g. starch/l., the 2nd 3 g./l. galactose, the 3rd sucrose and the 4th maltose. Similar comparisons of the influence of starch with that of other carbohydrates were also made. Thus the protein synthesis obtained in every instance could be related to that obtained with starch. Only the average amounts of synthesis in this first experiment are shown in Table 10, as the average values are so typical of all the results that there is no necessity to quote the individual values. But for Exps. 2–4 (Table 10) in which 7 incubations were carried out at one time, the individual results are quoted. In all these experiments no urea was included in the diet of the steer, and since in Exp. 1 (Table 10) none was added to the rumen liquor, the initial N.P.N. was very low, 4–10 mg. N/100 g. In Exps. 2–4, however, the N.P.N. was much higher, due to the addition of about 100 mg. urea-N/100 g. rumen liquor in each incubation. Presumably the greater amount of synthesis in the three latter experiments was due to this. The results, which confirm the fact that synthesis and hydrolysis are progressing simultaneously, but that synthesis predominates under certain conditions, lead to the following conclusions. (1) That the amount of synthesis depends to some extent on the starch

Table 10. *Protein synthesis in presence of carbohydrates*

Carbohydrate	Wt. added g./l.	Exp. 1: Average synthesis (+) or hydrolysis (−) mg. N/100 g.	Exp. 1: No. of determinations	Synthesis (mg. N/100 g.): Exp. 2	Exp. 3	Exp. 4	Final *p*H*
Starch	10	+3·7	2	—	—	13·7	7·0
,,	7	+3·5	2	—	—	13·3	6·9
,,	3	+4·0	18	13·7	14·1	10·9	6·9
,,	1	+1·9	2	—	—	5·7	6·9
,,	0·5	−1·9	2	—	—	4·5	6·6
No addition	—	−3·3	6	—	0·2	−2·0	6·4
Galactose	3	+3·5	5	10·5	10·5	—	—
Sucrose	3	+1·3	5	9·6	—	—	—
Maltose	3	+3·7	4	9·4	9·3	—	—
Lactose	3	+2·7	3	3·2	—	—	—
Glucose	3	+1·6	4	3·5	3·4	—	—
Dextrin	3	+0·5	5	—	0·1	—	—
Glycerol	3	−0·4	6	3·7	—	—	—
Lactic acid	3	−2·0	3	—	1·6	—	—

* The initial *p*H was 7·0 in all incubations.

concentration, e.g. in the absence of starch an hydrolysis of 2·0 mg. N/100 g. was observed, but in its presence synthesis predominated. In Exp. 4 protein synthesis attained a maximum at a starch concentration of 7 or 10 g./l. On the average, however, the synthesis with 7 and 10 g. starch/l. rarely exceeded that obtained with 3 g./l. Below 3 g./l. synthesis decreased significantly. (2) That the amount of synthesis depends on the nature of the carbohydrate added, being always greatest with starch, galactose and maltose; dextrin, glucose, glycerol and lactic acid being all relatively poor stimulants. (3) That with all these substances protein synthesis was much greater with, than without, urea.

Influence of amino-acids and proteins. In this section only preliminary experiments have been completed. The results are therefore presented tentatively. Rumen liquor was incubated with various amino-acids and proteins. In all instances 3 g. starch/l. were present and CO_2 was passed through the medium. The period of incubation was 3 hr. The data for Exp. 1 (Table 11) show that arginine was particularly active in promoting synthesis, whereas tyrosine had little, if any, effect.

Table 11. *Protein synthesis or hydrolysis in presence of amino-acids*

	Synthesis mg. N/100 g. rumen liquor		
No. of exp. ...	1	2	3
No addition	4·2	3·8	2·3
Glycine	6·0	—	—
Alanine	—	4·3	—
Valine	—	3·5	5·6
Leucine	8·9	—	—
Lysine	—	1·0	4·6
Histidine	7·9	1·8	—
Arginine	13·2	—	—
Tyrosine	5·5	—	—

Table 12. *Protein synthesis or hydrolysis in presence of leucine and asparagine*

Leucine		Asparagine	
Added %	Synthesis (+) or hydrolysis (−) mg. N/100 g.	Added %	Synthesis (+) or hydrolysis (−) mg. N/100 g.
None	+2·7	None	+4·8
0·47	−0·7	0·62	+1·1
1·13	−2·0	1·25	−4·5
2·26	−0·7	2·5	−1·2
0·47 + 0·5 urea	+6·6		
0·5 urea alone	+3·0		

How far this is due to arginase activity in the rumen will be investigated in future work. Histidine and leucine were effective but much less so than arginine. These results, however, were not confirmed in the two subsequent experiments. Thus histidine appeared to cause a decrease in protein synthesis (Table 11) and leucine actually brought about hydrolysis unless urea was present with the amino-acid (Table 12). The explanation is not at present clear. Perhaps some amino-acids only promote synthesis in certain samples of rumen liquor or when certain other non-protein nitrogenous constituents are present. Further work may clear this up. Table 12 shows that asparagine causes hydrolysis rather than synthesis.

Table 13. *Protein synthesis or hydrolysis in presence of protein or substances rich in protein*

Protein added %		Synthesis (+) or hydrolysis (−) mg. N/100 g. rumen liquor	
		Sample 1	Sample 2
None	—	+5·0	+ 4·0
Blood meal	0·1	+5·2	—
Egg white	—*	+1·0	—
Casein	0·1	−6·4	—
Gelatine	0·2	—	−12·5
Gelatine	0·5	—	−27·2
Gelatine	1·0	—	−47·0

* Equivalent to about 0·1% albumen.

In two other experiments with casein in which different samples of rumen contents were used, hydrolysis was 3·5 and 31·3 mg. N/100 g. compared with synthesis of 7·7 and 10·4 mg. N/100 g. respectively in the controls.

The results of preliminary experiments with four proteins are recorded in Table 13. With blood meal the amount of synthesis was the same as that in the absence of added protein. Fresh uncoagulated egg white gave a much reduced synthesis, whereas with soluble casein, prepared freshly from milk, hydrolysis predominated. Hydrolysis also prevailed when gelatine was present and increased with increasing amounts of this protein.

DISCUSSION

The object of this work has been to find whether there is any evidence for the conversion of N.P.N. to protein in the rumen ingesta of the ox. It has been shown that when the rumen ingesta are incubated *in vitro* the total N.P.N. usually diminishes during the first 3 hr. and that the decrease is caused entirely by a fall in the ammonia-N fraction of the N.P.N. It has not yet been proved that this decrease in ammonia-N actually denotes a synthesis of protein, but a decrease in N.P.N. with no change in the total N is usually regarded as evidence of protein synthesis. It has been shown that this supposed synthesis is characteristic of rumen contents but not of incubations with faeces, soil or feeding-stuffs,

and that it occurs *in vitro* during the first few hours of incubation when, as shown by Baker [1942 *a*, *b*; 1943], the microbiology of the medium closely resembles that of fresh rumen contents.

That this synthesis is microbiological in origin has been shown by incubations with various toxic substances such as NaF, quinone and boric acid in minute amounts which caused a decrease in synthesis which changed to hydrolysis as the concentration of the inhibitor increased; with the highest concentrations both synthesis and hydrolysis were inhibited. This indicates that in the rumen synthesis and hydrolysis of protein proceed simultaneously, and that either one or the other may predominate according to the conditions. Abundance of starch, for example, favours synthesis, whereas its equivalent of glucose, glycerol or lactic acid does not. Some substances, e.g. lactic acid and gelatine, may even promote conditions in which hydrolysis predominates. In such instances, however, hydrolysis is not accompanied by destruction of the urease activity of the rumen ingesta. This occurs only when hydrolysis predominates owing to the presence of small concentrations of toxic substances such as NaF, quinone or boric acid.

While the present work was in the early stages Wegner *et al.* [1940] demonstrated the conversion of inorganic N to protein by inoculating appropriate media with micro-organisms from the cow's rumen. But we have studied this problem not by the inoculation of media in which conditions may differ markedly from those in the rumen but by incubating the rumen liquor itself under conditions as similar as possible to those in the rumen. This was done originally because many of the micro-organisms most characteristic of the rumen do not survive in other media, and it seemed possible that these less viable organisms might be concerned in the utilization of N.P.N. Thus it was observed that on incubating rumen ingesta synthesis of protein appears to be accompanied by protein breakdown. Hence, it is clear that the problem of N.P.N. utilization by ruminants can ultimately be solved only when the different factors influencing these two processes have been studied. The centrifuging experiments (p. 158) strongly suggest that protein decomposition is associated particularly with the heavier micro-organisms which sediment rapidly, i.e. synthesis greatly predominates in the top layers of the centrifuged rumen liquor.

That either protein synthesis or protein breakdown may predominate, according to the conditions in the rumen ingesta, gives rise to at least two important considerations. (1) That N.P.N. may be of value to the animal only if it is fed in a correctly balanced diet in which the starch equivalent is sufficiently high to balance almost all the N present. (2) That for ruminants the biological value of a protein may depend not only on its amino-acid content but also on whether its presence in the paunch promotes protein synthesis or hydrolysis. Thus with blood meal, which is very insoluble in the rumen liquor and can be seen apparently intact in the rumen ingesta some hours after feeding, synthesis occurred in the *in vitro* incubations, but with other water-soluble proteins less protein synthesis, and even marked production of NH_3, took place. Possibly therefore blood meal, due to its insoluble nature, passes through the rumen practically unaffected and is digested in the small intestine as with non-ruminant animals, whereas other more soluble forms of protein may be broken down slightly but significantly to NH_3 as they pass through the rumen. To what extent a protein is broken down to NH_3 may partly depend on the nature and amount of the non-protein nitrogenous substances present at the time. Thus in the presence of leucine only (Table 11) slight hydrolysis to NH_3 took place, but when urea was also present synthesis predominated. Note that these suggestions are based on only one experiment and that they therefore require confirmation. Nevertheless such indications did appear from time to time. The value of N.P.N. to the ruminant may therefore be, that though it can be utilized for protein synthesis, under certain conditions it also diminishes protein breakdown.

SUMMARY

1. Many experiments have been carried out in which liquid rumen contents have been incubated under different conditions and with different substances.

2. During a 2–4 hr. incubation, when the microbiology appeared to be practically unaltered, the N.P.N. of the medium diminished while the total N remained unchanged, which suggests that synthesis of protein took place.

3. The amount of synthesis occurring in 2 hr. in the presence of 0·3 g. starch/100 g. rumen liquor was usually equivalent to about 8 mg. N/100 g. rumen liquor. If this rate were maintained for 24 hr. in the inact rumen there should be protein synthesis equivalent to 72 g. N or 450 g. protein.

4. That the synthesis is microbiological in nature has been shown by experiments with NaF, quinone and boric acid, and by incubations at different temperatures.

5. Relative amounts of synthesis in the presence of various carbohydrates have been studied. Starch appears to have the most stimulating effect.

6. Similar but preliminary experiments have been carried out with proteins and amino-acids.

7. Protein synthesis occurring during the incubation of rumen liquor is accompanied by protein breakdown. Either process may predominate ac-

cording to the general conditions or to the substances present. The possible bearing of this on the utilization of N.P.N. and on the biological value of proteins for ruminants is briefly discussed.

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